

February, 1953

Price 35s. net

VOL. 8, No. 1

THE JOURNAL OF GENERAL MICROBIOLOGY

EDITED FOR
THE SOCIETY FOR GENERAL MICROBIOLOGY

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CAMBRIDGE UNIVERSITY PRESS

LONDON: BENTLEY HOUSE, N.W.1

NEW YORK: 32 EAST 57TH STREET, 22

Reprinted by offset-litho 1954

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VOLUME 8, 1953

CAMBRIDGE
AT THE UNIVERSITY PRESS
1953

*First Printed in Great Britain at the University Press, Cambridge
Reprinted by offset-litho by Jarrold & Sons Ltd., Norwich
and published by the Cambridge University Press
Cambridge, and Bentley House, London
Agents for Canada and India: Macmillan*



Rn 2770/ZB

(PC / Prof. Hantel)

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DAGLEY, S., FEWSTER, M. E. & HAPPOLD, F. C. (1953). *J. gen. Microbiol.* 8, 1-7.

The Bacterial Oxidation of Aromatic Compounds

By S. DAGLEY, MONA E. FEWSTER AND F. C. HAPPOLD

Department of Biochemistry, University of Leeds

SUMMARY: Adaptive patterns for a vibrio indicate that the oxidation of phenylalanine to homogentisic acid by this organism may proceed by two different pathways, one through phenylpyruvic and phenylacetic acids and the other through tyrosine and *p*-hydroxyphenylpyruvic acid. That the former pathway is used is confirmed by the isolation from metabolism fluids of the phenylhydrazone of phenylpyruvic acid. The vibrio does not appear to oxidize the side chains of phenylpropionic and phenylacetic acids before ring fission. The influence of cell suspension density on rates of oxidation of various highly polar compounds which may penetrate slowly into the cells has been studied.

The technique of simultaneous adaptation is of particular value in elucidating the pathways of bacterial oxidation of aromatic compounds (Stanier, 1947; Suda, Hayaishi & Oda, 1950; Karlsson & Barker, 1948). These studies have been principally concerned with the breakdown of mandelic and benzoic acids, phenol and other compounds, but comprehensive studies of the oxidation of phenylpropionic acid, phenylacetic acid, tyrosine or phenylalanine have not been reported. In the present work we measured rates of oxidation of some of the compounds whose presence in growing cultures of a vibrio has been claimed (Jones, Smith & Evans, 1952) and for those known to be of significance in mammalian metabolism.

METHODS AND MATERIALS

The organism used throughout this work was the vibrio 01 originally isolated by Happold & Key (1932). It is capable of oxidizing various phenols, substituted benzoic acids and aromatic acids (Evans, 1947).

The medium used was as follows: $(\text{NH}_4)_2\text{SO}_4$, 1 g.; KH_2PO_4 , 2.5 g.; MgCl_2 , 0.5 g.; FeCl_3 , 2 mg. made to 1 l. with glass-distilled water. The appropriate carbon source was added to give a final concentration of 0.01 M and the pH adjusted to 7.2. The addition of MgCl_2 was made aseptically after autoclaving. Cells able to metabolize the various carbon sources were obtained by at least three preliminary serial subcultures in the required medium which was aerated by a gentle stream of sterile air and incubated at 30°. The organisms were then harvested by centrifugation after overnight incubation of the cultures. Suspensions were prepared in 0.067 M-phosphate buffer (pH 7.0) after washing the cells twice with distilled water.

Oxygen uptakes were measured using the Warburg apparatus. Experiments were conducted at 30° in an atmosphere of air using 1.0 ml. of cell suspension and 1.4 ml. of 0.067 M-phosphate buffer (pH 7.0); 0.4 ml. of 0.01 M substrate was usually added from the side arm; 0.2 ml. (10%) KOH was used in the centre well. In all cases, the total volume of fluid in each flask was 3.0 ml.

The low solubility of tyrosine necessitated a modification of procedure; 1.6 ml. of 0.0025M-tyrosine solution was placed in the flask and 0.5 ml. cell suspension added from the side arm.

Some of the materials used were not available commercially. Phenylpyruvic acid was synthesized as the sodium salt according to the method of Corson, Dodge, Harris & Hazen (1944), and benzoylformic acid as described by Herbst & Shemin (1944).

RESULTS

Cell permeability to different substrates

Although β -ketoadipic acid has been shown to be an intermediate in the oxidation of mandelic acid by *Pseudomonas fluorescens* (Stanier, Sleeper, Tsuchida & Macdonald, 1950), and by vibrio 01 (Kilby, 1948, 1951), it is oxidized at a much slower rate than mandelic acid (Stanier, 1950). Stanier has attributed this to the slow penetration of the highly polar β -ketoadipic acid through the cell membrane. Several of the compounds we investigated contain two or more polar groups, and the rate at which they were oxidized was small. It was difficult to decide whether the initial rate of oxidation of these compounds was significantly greater than the endogenous oxygen uptake and hence to assess their roles as possible intermediates. This difficulty cannot well be overcome by increasing the substrate concentrations of compounds only available in very small quantities; alterations of the pH value of the mixtures over the range 6.5–8.0 were without significant effect on the oxidation rates. Variations in density of cell suspensions, however, resulted in disproportionate effects on rates of oxidation of the highly polar compounds relative to the rates for their assumed precursors. This effect is illustrated by the following figures for *cis-cis* muconic acid, which was claimed by Evans & Smith (1951) to be an intermediate in the oxidative breakdown of mandelic acid. With 5 mg. dry-weight cells/flask grown on mandelic acid medium, the initial rates of oxygen uptake were 13.9, 1.2 and 0.35 $\mu\text{l./min.}$ for mandelic acid, *cis-cis* muconic acid and cells in the absence of substrate, respectively. When the cell density was increased to 20 mg. dry weight/flask the corresponding values were 15.5, 5.9 and 1.8 $\mu\text{l./min.}$, showing a fivefold increase for the rate of oxidation of *cis-cis* muconic acid and for the endogenous respiration, and only a 10% increase for mandelic acid. Therefore, whenever a result was difficult to interpret on account of a low oxidation rate, the work was repeated using higher cell densities.

Comparison of rates of oxidation of various substrates by cells grown on phenylalanine, tyrosine and phenylpyruvic acid

Fig. 1 shows the influence of the nature of the carbon source utilized in the growth medium on the ability of the washed cells to oxidize certain compounds. Phenylalanine-grown cells oxidized phenylpyruvic and phenylacetic acids at approximately the same rate as phenylalanine. Tyrosine-grown cells, however, did not readily oxidize these substrates; for phenylpyruvate an adaptive curve was obtained, and for phenylacetate a curve indistinguishable from the

autorespiration. This suggests that phenylpyruvic and phenylacetic acids may be intermediates in the oxidation of phenylalanine but not of tyrosine. The complete adaptation of phenylpyruvic acid-grown cells to phenylacetic acid (Fig. 1) is consistent with this suggestion. Cells grown on phenylalanine were fully adapted to tyrosine but the reverse was not true. Benzoic acid does not

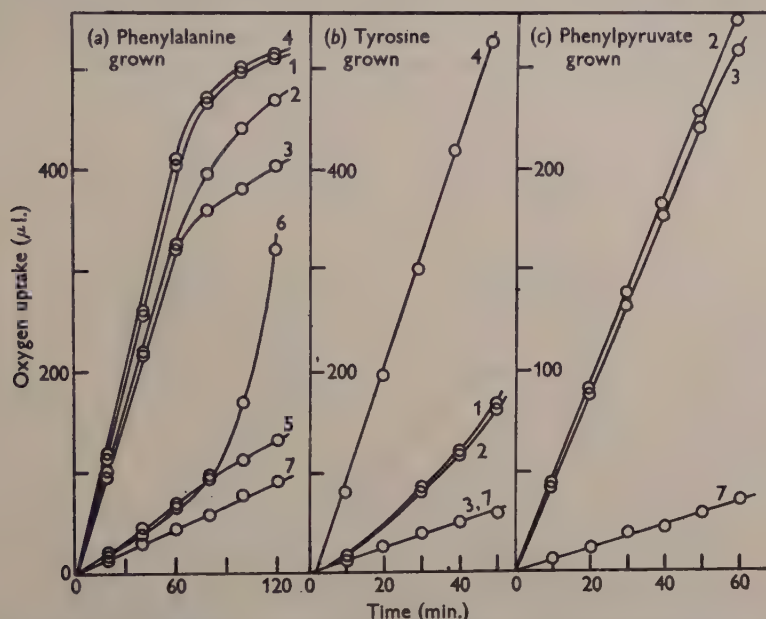


Fig. 1. The effect of conditions of growth on adaptive patterns with respect to the following compounds: 1, DL-phenylalanine; 2, phenylpyruvic acid; 3, phenylacetic acid; 4, L-tyrosine; 5, phenylpropionic acid; 6, benzoic acid; 7, autorespiration. Each vessel contained c. 5 mg. dry weight of cells and in (a) 4 μ M. of the respective substrate, in (b) and (c) 5 μ M.

appear to be an important intermediate in the oxidation of phenylalanine by the vibrio 01, since the curve (Fig. 1a) obtained with this substrate was typically adaptive. Phenylpropionic acid was not readily metabolized by cells grown on phenylalanine.

Comparison of rates of oxidation of various substrates by cells grown on phenylpropionic, phenylacetic and mandelic acids

With *Ps. fluorescens*, Stanier (1947, 1950) found that cells grown on a mandelic acid medium were fully adapted to benzoylformic and benzoic acids, whilst those grown on phenylacetic acid were not. In the present work we obtained similar results with cells of vibrio 01; cells grown on phenylacetic acid were not adapted to benzoylformic acid. Similarly, we obtained no evidence that the side chain of phenylpropionic acid was attacked prior to ring fission, since cells grown at the expense of this compound were unable to oxidize phenylpyruvic, phenylacetic or benzoic acids without adaptation.

Comparison of rates of oxidation of highly polar compounds by cells grown on tyrosine, phenylalanine and phenylacetic acid

The necessity for using denser cell suspensions with highly polar substrates has already been discussed. The use of suspensions of tyrosine-grown cells of approximately the same density as in previous experiments did not permit definite conclusions as to the oxidation of *p*-hydroxyphenylpyruvic, homogentisic and *p*-hydroxyphenylacetic acids, since the curves differed only

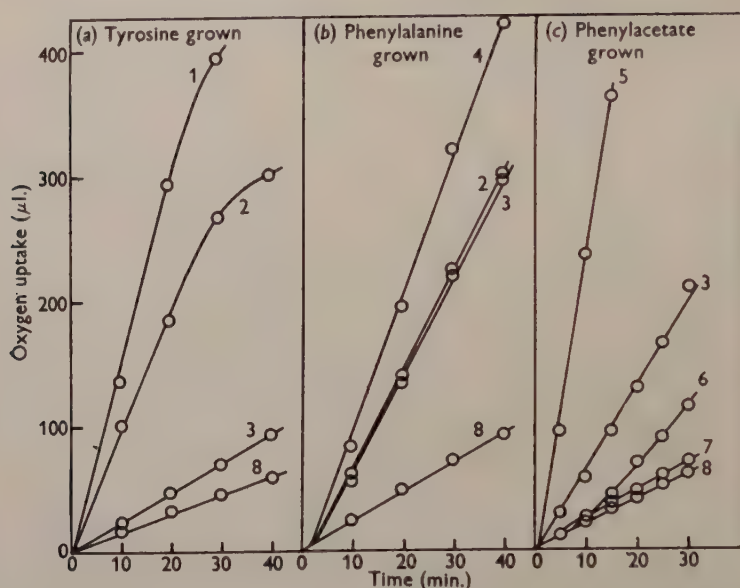


Fig. 2. Oxygen uptake in the presence of the following: 1, L-tyrosine; 2, *p*-hydroxyphenylpyruvic acid; 3, homogentisic acid; 4, DL-phenylalanine; 5, phenylacetic acid; 6, *p*-hydroxybenzoic acid; 7, *p*-hydroxyphenylacetic acid; 8, autorespiration. Each vessel contained 15 mg. dry weight of cells and 4 μ M. of the respective compound.

slightly from that for autorespiration. When the cell density was increased threefold (c. 15 mg. dry weight/flask), *p*-hydroxyphenylpyruvic acid was readily oxidized and homogentisic acid was oxidized at a linear but much slower rate (Fig. 2a). Towards the conclusion of this work, we learnt that Jones *et al.* (1952) had found that cells grown on tyrosine media were completely adapted to oxidize homogentisic acid. Using *p*-hydroxyphenylacetic acid, the initial rate of oxygen uptake was low but when the experiment was continued for a period longer than 40 min. the rate increased and an adaptive curve was obtained (Fig. 3).

Using strong suspensions (approximately 15 mg. dry weight/flask) of cells grown on phenylalanine medium, *p*-hydroxyphenylpyruvic and homogentisic acids were readily oxidized (Fig. 2b). Adaptive curves were obtained using *p*-hydroxybenzoic and *p*-hydroxyphenylacetic acids (Fig. 3). Suspensions of

phenylacetate-grown cells of similar density were completely adapted to homogentisic acid but not to *p*-hydroxybenzoic or *p*-hydroxyphenylacetic acids.

*Isolation of phenylpyruvic acid phenylhydrazone
from metabolism fluid*

Each of ten 100 ml. batches of a 1% solution of DL-phenylalanine in phosphate buffer (pH 7.1) was aerated at 30° with *c.* 400 mg. dry weight of washed cells. The total quantity of cells (*c.* 4 g. dry weight) for this experiment was harvested from ten 1 l. batches of phenylalanine medium separately

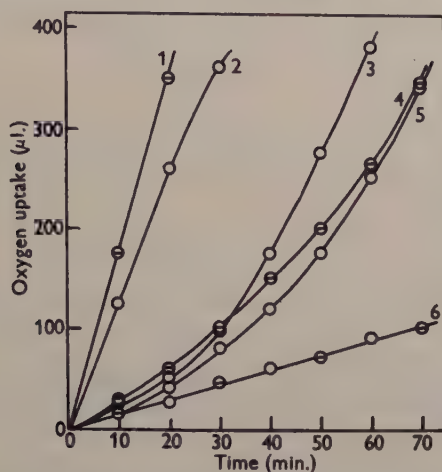


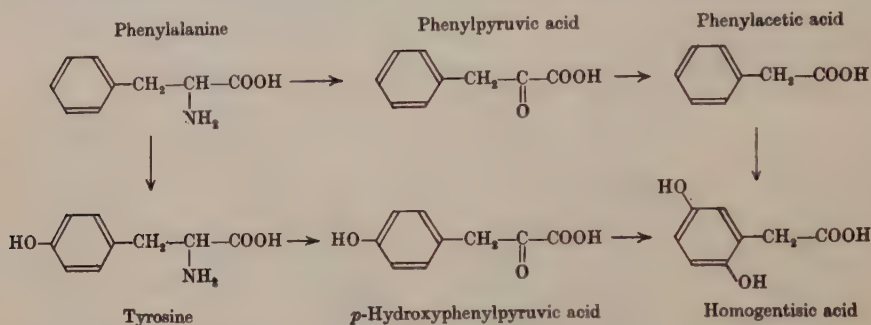
Fig. 3. Oxidation of *p*-hydroxybenzoic and *p*-hydroxyphenylacetic acids. Vessels contained *c.* 15 mg. dry weight of cells grown in: ○, phenylalanine medium; ⊖, tyrosine medium. Curves show oxygen uptake in presence of 4 μM. of (1) L-tyrosine; (2) DL-phenylalanine; (3) *p*-hydroxybenzoic acid; (4) and (5) *p*-hydroxyphenylacetic acid; (6) no added substrate.

aerated and incubated at 30°. Addition of ferric chloride solution to samples withdrawn at intervals gave a green colour characteristic of phenylpyruvic acid. Aeration was continued until maximum coloration was produced, when the suspensions were centrifuged, Seitz-filtered and the filtrate concentrated at 37° *in vacuo*. After acidification and addition of phenylhydrazine hydrochloride in sodium acetate solution, a pale yellow precipitate was obtained which after recrystallization from an ethanol+water mixture, melted at 155°, alone or admixed with the phenylhydrazone of authentic phenylpyruvic acid. Analysis (Drs Weiler and Strauss, Oxford) gave: C, 70.9; N, 10.7; H, 5.9; $C_{15}H_{14}O_2N_2$ requires C, 70.9; N, 11.0; H, 5.5.

DISCUSSION

The results shown in Fig. 1 are compatible with an oxidation pathway of phenylalanine by way of phenylpyruvic and phenylacetic acids; this suggestion is confirmed by our observation of the formation of phenylpyruvic acid from phenylalanine by suspensions of the vibrio. Since these cells are also completely

adapted to oxidize tyrosine, while cells from tyrosine medium are not adapted to phenylalanine, an alternative pathway for the oxidation of the latter by way of tyrosine is also possible. This second route was suggested by Evans, Smith, Linstead & Elvidge (1951) and is supported by our observations (Fig. 2) that cells grown on phenylalanine and tyrosine media are both simultaneously adapted to *p*-hydroxyphenylpyruvic acid, in contrast to the behaviour of tyrosine-grown cells towards phenylpyruvic acid. These alternative routes are summarized below:



The detection of phenylpyruvic acid during oxidation of phenylalanine by the vibrio and isolation of the phenylhydrazone, uncontaminated with *p*-hydroxyphenylpyruvic phenylhydrazone, is some indication that oxidative deamination of the amino-acid constitutes the main pathway in the vibrio rather than tyrosine formation as postulated by Evans *et al.* (1951) for this vibrio and for *Ps. fluorescens*.

The suggestion of *p*-hydroxyphenylacetic acid as a precursor of homogentisic acid is not supported by our results with vibrio 01 since neither tyrosine-grown nor phenylacetate-grown cells were adapted to *p*-hydroxyphenylacetic acid. The above scheme for the oxidation of phenylalanine to homogentisic acid by way of tyrosine is similar to that postulated for mammalian metabolism (Rimington, 1950). Kluyver & van Zijp (1951) demonstrated the production of homogentisic acid from phenylacetic acid by *Aspergillus niger*. Jones *et al.* (1952) showed chromatographically the presence of the following substances in the culture fluid of this vibrio, and of strains of *Ps. fluorescens* growing at the expense of tyrosine: homogentisic, *p*-hydroxyphenylpyruvic, *p*-hydroxyphenylacetic and *p*-hydroxybenzoic acids. They also showed that cells grown on tyrosine media were completely adapted to oxidize homogentisic acid. The present evidence indicates that *p*-hydroxybenzoic acid is not an important intermediate in the oxidation of phenylalanine, tyrosine or phenylacetic by vibrio 01; and vibrios from tyrosine or phenylacetate media are not simultaneously adapted to *p*-hydroxyphenylacetic acid. It is possible that substances found in growing cultures which do not satisfy the simultaneous adaptation criteria as intermediates may be end products rather than oxidative intermediates, or their presence may indicate the existence of a shunt mechanism (Happold, 1950). Whilst the present work shows the value of applying adaptation criteria alongside attempts to isolate intermediates,

it also emphasizes that measurements of oxygen uptake for highly polar substrates must be interpreted with particular caution.

Experiments with phenylpropionic and phenylacetic acids show that ring fission by vibrio 01 does not precede degradation of the side chain. When, however, there is a substituent in the latter, as in phenylalanine, tyrosine, phenylpyruvic acid or mandelic acid, the side chain is the first to undergo attack.

We are indebted to the Agricultural Research Council for a grant to this Department; to Dr A. Neuberger, F.R.S., who kindly supplied us with samples of homogentisic and *p*-hydroxyphenylpyruvic acids; to Prof. R. P. Linstead, F.R.S., for a sample of *cis-cis* muconic acid. One of us (M. E. F.) acknowledges the award of a bursary by the South African Council for Scientific and Industrial Research.

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(Received 7 February 1952)

An Adaptive Bacterial Cholinesterase from a *Pseudomonas* Species

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SUMMARY: An organism belonging to the *Pseudomonas fluorescens* species-group has been isolated from fermenting cucumber by enrichment culture, using acetylcholine as the sole carbon source. This organism produces an adaptive cholinesterase.

Cells grown in tryptic digest broth have a very low cholinesterase activity. Growth for a few days in the presence of acetylcholine, choline, or Difco nutrient broth produces much higher cholinesterase levels. Cell-free preparations are also active.

The enzyme is not identical with either of the main types of animal cholinesterase. It is sensitive to prostigmine, is not inhibited by excess of substrate, and has a unique substrate specificity pattern.

The organism also produces two prostigmine-insensitive lipases, splitting triacetin and tributyrin, respectively.

Cholinesterases belonging to two major types have been found in a large number of animal species (Augustinsson, 1948). The nerve-type acetylcholinesterase is associated only with specific organ systems (nervous tissue, erythrocytes) which are limited to the animal kingdom. Its function in nervous tissue has been partially elucidated. The function of the plasma-type cholinesterase found in plasma and various organs remains obscure. Do similar enzymes exist in micro-organisms? If so, what is their function in the microbial cell, and are they identical with either of the main types of animal cholinesterase?

Previous attempts to find cholinesterases in bacteria have been made by testing certain pathogens for their ability to split acetylcholine. Esterases have been found in pneumococci (Bernouilli & Bloch, 1944), and one strain has been shown to hydrolyse acetylcholine, but the specificity of the enzyme has not been explored (Schaller, 1942). Several other micro-organisms have shown very low cholinesterase contents (Vincent & de Prat, 1945; de Prat, 1945).

The enrichment culture technique seems more promising because it allows one to select from a large number of bacterial species those capable of utilizing acetylcholine as a carbon source for growth. Some of these may be equipped with cholinesterase, while others may attack the acetylcholine molecule otherwise than by preliminary hydrolysis. Not all organisms possessing cholinesterase would be isolated, since those which require specific growth substances other than acetate and choline would not survive in the enrichment culture.

A suitable starting material for such an enrichment culture would be one

in which acetylcholine is present. Fermenting plant materials are known to contain acetylcholine, and Stephenson & Rowatt (1947) have reported the isolation from sauerkraut of lactobacilli capable of synthesizing acetylcholine. It seemed likely that such a natural environment might also harbour organisms able to utilize acetylcholine as a nutrient. In these experiments fermenting cucumber was used to start the enrichment culture.

METHODS

Isolation. Mashed cucumber was allowed to ferment in a closed bottle filled to the brim with tap water. Vigorous gas production was seen in a few days and the medium became acid. On the third day a transfer was made to a 25 ml. Erlenmeyer flask containing 0.1 % recrystallized acetylcholine (ACh) in 5 ml. of a mineral medium consisting of KH_2PO_4 0.1 %, NH_4Cl 0.1 %, and MgSO_4 0.05 %, at pH 6. After a second transfer in the same medium, the culture was plated out on Difco nutrient agar. All cultures were grown aerobically at room temperature. Solutions containing ACh were sterilized by filtration.

Growth and respiration. Growth experiments to determine which substrates could serve as carbon or nitrogen source were carried out in mineral medium with ACh bromide, sodium acetate, choline chloride, or glucose, added at 0.1 % concentration. When the substrate was to be tested as a nitrogen source, 0.1 % NaCl was substituted for the NH_4Cl in the mineral medium.

Respiration studies were done in the Warburg manometer at 27° with 20 % KOH in the centre well. The cells were harvested from nutrient agar plates and suspended in tap water. 0.2 ml. of the substrate was added from the sidearm after the rate of endogenous respiration had been established.

Esterase determinations were done manometrically at 27° in an atmosphere of 95 % N_2 + 5 % CO_2 . Intact cells or acetone-dried powders were suspended in bicarbonate Ringer solution (NaCl, 9 g.; KCl, 0.3 g.; CaCl_2 , 0.25 g.; NaHCO_3 , 2.1 g./l.; this solution is buffered to pH 7.3 when equilibrated with the gas mixture); 0.2 ml. of ACh or other ester was added from the sidearm. When prostigmine was used, it was added to the enzyme preparation in the vessel and incubated for at least 2½ hr. before addition of substrate.

Cell-free enzyme preparations. Cells were grown at room temperature in Roux bottles containing tryptic digest broth (beef heart digested with trypsin and filtered, with 0.1 % glucose added). Growth was heavy after 40 hr., and the culture had apparently reached the stationary phase of its growth. Acetone-dried powders were prepared by centrifuging the broth cultures and resuspending the cells twice in a large volume of acetone at 0°. The final centrifugate was allowed to dry at room temperature and ground in a mortar to a fine powder. The yield was 1.5–2 g./l. of broth. Weighed portions of powder (usually 50 mg.) were placed in Warburg vessels. With this weight of powder, duplicates gave good checks, but with larger amounts the suspensions were sometimes inhomogeneous. The powders have the disadvantage that there is considerable endogenous acid production before the ACh is added, but this could be decreased by incubation of the powder with the Ringer

solution for a few hours. Attempts to get the enzyme into solution from these preparations were abandoned because, on addition of water, a thick mucoid paste was formed, which could not easily be filtered or centrifuged.

RESULTS

Isolation and characterization. An organism was isolated on nutrient agar, which subsequently grew on ACh in mineral medium. It is a small, motile, Gram-negative rod, which grows on nutrient agar as a small, pale green, moist colony producing a green fluorescent pigment soluble in the medium.

Table 1. *Behaviour of the organism in routine tests*

Gelatin liquefaction	+	Gas production from sugars	—
Coagulation of milk	—	Indole production	—
Acid production from		NO ₃ reduction to NO ₂	+
Glucose	+	Growth on citrate	+
Lactose	—	Methyl red	—
Sucrose	—	Voges-Proskauer	—
Mannitol	—		
Salicin	—		

Pyocyanine was not detected when cultures grown on various media were extracted with chloroform in which pyocyanine is soluble but the fluorescent pigment is not. The organism grows well at room temperature but not at all at 37°. Further classification tests are summarized in Table 1. The organism is a member of the *Pseudomonas fluorescens* species group (Stanier, 1947). It has been deposited in the American Type Culture Collection (ATCC 11150) and the National Collection of Industrial Bacteria (NCIB 8286).

Growth and respiration. Media containing acetate as carbon source and ammonia as nitrogen source did not support growth. Choline could be utilized as both carbon and nitrogen source. Glucose is a satisfactory carbon source, though in unbuffered media the total growth is limited.

Fig. 1 shows that a suspension of the bacteria can oxidize ACh, as well as acetate and choline. ACh was utilized at the rate of about 0.6 μ mole ACh/hr. Since the spontaneous hydrolysis rate is less than 0.2 μ mole/hr., the organism could not have been merely oxidizing the products of spontaneous hydrolysis.

In the run shown in Fig. 1, 8 mole of oxygen were taken up per mole of ACh present, on the assumption that the total observed rate represents the sum of the endogenous rate and that due to ACh oxidation. In other experiments, values ranging from 6 to 8 mole of oxygen were obtained. Complete oxidation of ACh to CO₂ and NH₃ would use 9 mole of oxygen per mole of ACh.

Esterase

(1) *Demonstration of cholinesterase (ChE)*

Appreciable amounts of CO₂ are released from bicarbonate Ringer solution in the presence of ACh by suspensions of intact cells or cell-free preparations. Intact cells split about 2 μ mole of ACh/hr./50 mg. dry weight. Acetone-dried powders vary in activity from 1 to 5 μ mole of ACh split/hr./50 mg. The spontaneous hydrolysis rate of ACh was measured at concentrations of 0.02–2.0 %

and does not exceed $0.2 \mu\text{mole/hr}$. Both with cells and with powders, some 'endogenous' CO_2 release, usually less than $1 \mu\text{mole/hr}$., was seen before addition of the ester. Esterase activities, as given below, are corrected for this endogenous rate and for the spontaneous hydrolysis of the substrate.

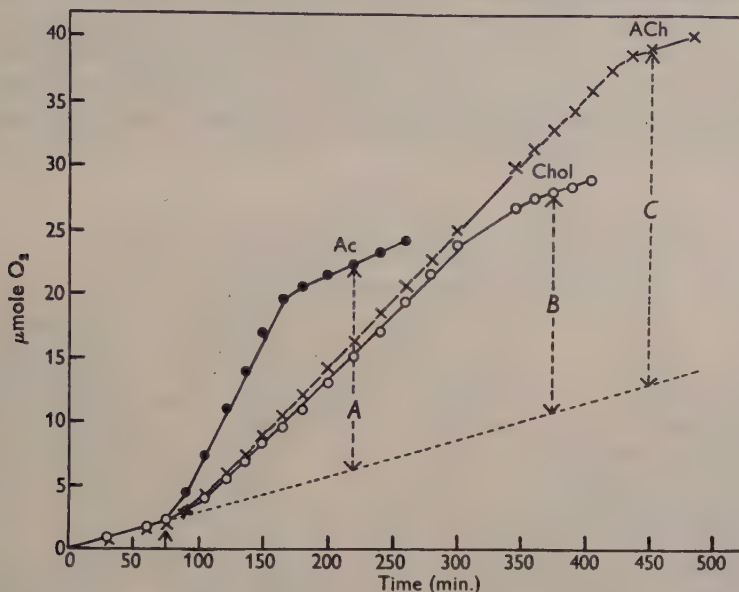


Fig. 1. Respiration of bacterial suspension in tap water. The following amounts of substrates were added at 75 min.: ACh bromide, $3.4 \mu\text{mole}$; choline chloride, $3.4 \mu\text{mole}$; sodium acetate, $17 \mu\text{mole}$. A, 1 mole O_2 : 1 mole Ac; B, 5 mole O_2 : 1 mole Chol; C, 8 mole O_2 : 1 mole ACh.

Is the ChE essential for the utilization of ACh as a nutrient? The relative rates of oxidation and hydrolysis of the molecule supply a clue, for only if the ester can be hydrolysed faster than it can be oxidized may we postulate that hydrolysis is a necessary first step in its metabolism. Aliquot portions of a suspension of intact cells were tested with the following results:

Hydrolysis: $1.1 \mu\text{mole ACh hydrolysed/hr}$.

Oxidation: $6.3 \mu\text{mole O}_2$ taken up/hr. At 6–8 mole $\text{O}_2/\text{mole ACh}$ (see above) this represents $1.1\text{--}0.8 \mu\text{mole ACh oxidized/hr}$.

Thus it is reasonable to suppose that the organism attacks the ester by preliminary hydrolysis, followed by oxidation of the fragments.

(2) *Adaptation*

The esterase activity is determined in part by the medium in which the cells are grown. Initially, the organism was grown on nutrient agar, and active enzyme preparations were obtained. Later, tryptic digest media were used and no enzyme activity was found. However, the enzyme reappeared when the cells were grown in tryptic digest broth with added ACh (0.2%). Thereafter two separate cultures were maintained on tryptic digest agar, one with and one without added ACh.

The culture grown without ACh was used to test the time course of the adaptation (Table 2). After 48 hr. growth in broth without ACh an acetone-dried powder was prepared. It was inactive. This broth culture was used to inoculate flasks of ACh-containing broth from which powders were prepared at intervals during the next 3 days. In the first 24 hr. of growth there was hardly any enzyme activity, but in the course of the next 2 days the ChE appeared in amounts comparable to those of cultures grown for a long time on ACh media. Growth for 3 days in tryptic digest broth without ACh produced inactive preparations.

Table 2. *Adaptive cholinesterase activity of acetone-dried powders from cultures grown in various media*

ACh concentration in the esterase determinations: 0.2%.

Medium	Run 1		Run 2		Run 3	
	Hours growth	Corr. rate*	Hours growth	Corr. rate	Hours growth	Corr. rate
Tryptic digest†	48	0.1	45	0.3	48	0.1
Tryptic digest + 0.2% ACh	18	0.1	18	0.2	24	0.3
Tryptic digest + 0.2% ACh	60	4.1	43	3.5	34	0.8
Tryptic digest + 0.2% ACh	.	.	67	2.1	48	1.2
Tryptic digest + 0.2% ACh	74	5.5
Tryptic digest + 0.2% choline	.	.	67	1.8	48	0.5
Nutrient broth	.	.	67	2.2	48	0.4
Nutrient broth + 0.2% ACh	.	.	67	9.7	48	6.2
Nutrient broth + 0.2% ACh	74	11.0

* Expressed as $\mu\text{mole CO}_2/\text{hr.}/50 \text{ mg. powder.}$

† These cultures were used to inoculate the others in the same run.

Nutrient broth (with no additions) or tryptic digest broth containing 0.2% choline were found to be almost as effective as ACh tryptic digest media in stimulating enzyme production. By far the most active preparations were obtained from nutrient broth with added ACh. Two cultures grown on ACh showed activities of 1.6 and 1.4 $\mu\text{mole CO}_2/\text{hr.}/50 \text{ mg. powder.}$ A single 48 hr. subculture without ACh decreased the activities to 0.4 and 0.1, respectively. It would of course have been interesting to follow enzyme production on mineral medium with ACh, but this has not been done because of the poor growth on this medium.

(3) *Substrate specificity*

(a) Cultures grown with ACh. The substrate specificity pattern was studied for comparison with those of other cholinesterases. Table 3 shows the behaviour of adapted acetone-dried powders. The simple esters methyl and ethyl acetate or butyrate were not attacked by the enzyme. Of the four choline esters tested, butyryl- and benzoylcholine were not split. Acetyl- β -methylcholine was hydrolysed slightly more slowly than ACh, and propionylcholine (PrCh) nearly 4 times as fast as ACh. Nu 2017-1 [(3-acetoxypheyl)-trimethyl ammonium iodide], an acetyl analogue of prostigmine, was split at about the

same rate as ACh. The structures of some of these compounds are shown in Fig. 2.

(b) Cultures grown without ACh. Unadapted acetone-dried powders showed no appreciable enzyme activity with ACh, acetyl- β -methylcholine, methyl

Table 3. *Substrate specificity*

Esterase activity of acetone-dried powders from cultures grown with ACh. Each powder was tested with ACh (0.2 % in the reaction mixture) and with one or more other substrates. The final column shows the relative rate of hydrolysis of each ester compared with ACh.

Substrate	Substrate % conc.	Spontaneous hydrolysis	Corrected rate $\mu\text{mole CO}_2/\text{hr.}$		Ratio: other substrate: ACh
			ACh	Other substrate	
Methyl acetate	0.1	0.1	2.8*	-0.2	Not split
Ethyl acetate	0.2	.	5.5	0.1†	Not split
Methyl butyrate	0.1	.	5.5	-0.3†	Not split
Ethyl butyrate	0.05	0.2	2.8*	-0.4	Not split
Acetyl- β -methylcholine Cl	0.2	0.4	3.8	2.0	0.6
PrCh Cl	0.2	0.1	3.9	14.6	3.7
PrCh Cl	0.2	0.1	1.8, 1.9	6.8, 7.3	3.8
PrCh Cl	0.2	0.1	7.0	27.8	4.0
Butyrylcholine Cl	0.2	0.1	3.9	-0.2	Not split
Butyrylcholine Cl	0.2	0.1	4.1	0.1	Not split
Benzoylcholine Cl	0.2	.	3.3	-0.4†	Not split
Nu 2017-1	0.2	0.2	3.3, 3.6	3.8, 3.8	1.1
Nu 2017-1	0.2	0.2	1.8, 1.9	2.9, 3.0	1.6
Triacetin	0.2	0.2	5.5	12.7	2.3
Triacetin	0.2	0.2	11.0	23.3	2.1
Tributylin	(10.0)‡	0.2	11.0	11.6	1.1

* ACh 0.1 % in the reaction mixture.

† Not corrected for spontaneous hydrolysis.

‡ The sidearm contained 0.2 ml. of tributyrin, a liquid poorly miscible with water.

butyrate or ethyl acetate as substrates. In one 10 hr. run, intact cells showed a small but definite activity towards both ACh and PrCh. The corrected rates of CO_2 evolution were constant ($0.46 \mu\text{mole CO}_2/\text{hr.}$ for ACh and 2.28 for PrCh), giving a ratio of 1:5 which is approximately the same as that shown by adapted powders.

(4) *Effect of varying substrate concentration*

The rate of CO_2 production was essentially constant at different ACh concentrations in the range 4.5×10^{-3} to $8.9 \times 10^{-2}\text{M.}$ No 'inhibition by excess of substrate' was seen. These results are shown in Table 4, which also includes similar data for PrCh. With the latter substrate, enzyme activity decreased about 25 % with increasing substrate concentration from 5×10^{-3} to 10^{-1}M.

(5) *Inhibition by prostigmine*

Prostigmine was added to acetone-dried powder suspensions in Ringer solution and allowed to incubate for several hours before testing. As shown in Table 5, the esterase activity was completely abolished by prostigmine concentrations of 10^{-3} to 10^{-2} M., using either ACh or PrCh as substrate; partial inhibition (68 %) was produced by 10^{-6} M. in intact adapted cells.

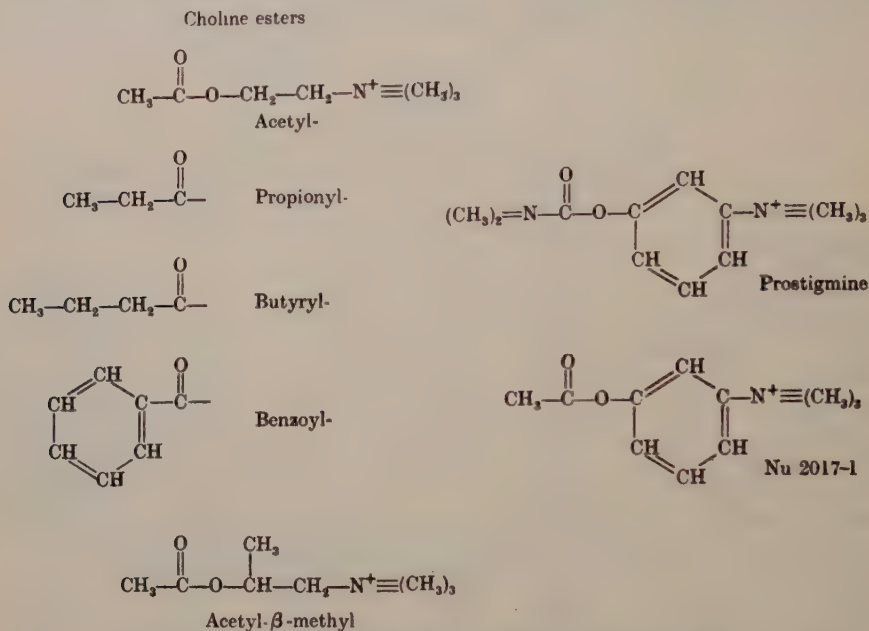


Fig. 2. Structures of choline esters, prostigmine, and Nu 2017-1.

Table 4. *Effect of varying substrate concentration on cholinesterase activities of acetone-dried powders from cultures grown with ACh*

Powder no.	Substrate	Concentration (M.)	Corrected rate ($\mu\text{mole CO}_2/\text{hr.}/50 \text{ mg. powder}$)
1	ACh Br	4.5×10^{-3}	4.4
	ACh Br	8.9×10^{-3}	4.1
	ACh Br	4.5×10^{-2}	4.1
	ACh Br	8.9×10^{-2}	4.0
2	PrCh Cl	5.0×10^{-3}	22.1
	PrCh Cl	1.0×10^{-2}	19.7
	PrCh Cl	5.0×10^{-2}	17.2
	PrCh Cl	1.0×10^{-1}	16.1

(6) *Lipase activity*

Triacetin and tributyrin were used as substrates for various enzyme preparations with the following results.

(a) Adapted acetone-dried powders split both fats. Tributyrin is split at about the same rate as ACh (Tables 3 and 5), but apparently by another enzyme

since it is insensitive to prostigmine (Table 5). Triacetin is split about twice as fast as ACh and the whole triacetinase activity is prostigmine-sensitive. A further indication that the triacetin is split here only by the ChE is provided by a 'mixed-substrate experiment' using ACh and triacetin. Here the corrected rates were: ACh alone $5.5 \mu\text{mole CO}_2/\text{hr.}$, triacetin alone 12.7, ACh + triacetin 5.9. As the rates are not additive, it would appear that in an adapted powder ChE is the main enzyme splitting triacetin.

Table 5. *Inhibition by prostigmine of cholinesterase activity of acetone-dried powders from cultures grown with ACh*

Run no.	Substrate	Prostigmine concentration (M.)	Incubation time (hr.)	Corrected rate ($\mu\text{mole CO}_2/\text{hr.}$)	
				Controls	With prostigmine
1	ACh	1.5×10^{-3}	18	2.5, 2.6	-0.3, 0.7
2	ACh	5×10^{-3}	4	7.0	0
	PrCh	5×10^{-3}	4	27.8	0
3	ACh	8×10^{-3}	3.5	11.0	-0.6
	Triacetin	8×10^{-3}	3.5	23.3	0.3
	Tributyrin	8×10^{-3}	3.5	11.6	11.4

Substrate concentrations in reaction mixture: ACh, PrCh, and triacetin 0.2%; tributyrin 0.2 ml. in sidearm.

(b) An unadapted acetone-dried powder (without ChE) failed to split triacetin. Thus no acetone-dried powder shows any triacetinase activity other than that due to ChE.

(c) Unadapted intact cells, however, split triacetin as well as tributyrin, and this triacetinase activity was unaffected by prostigmine. Measurement of total CO_2 evolution for known amounts of triacetin showed that only two of the three ester links were hydrolysed. Cells grown in tryptic digest broth with 0.2% triacetin split triacetin (prostigmine-insensitive) but not ACh, i.e. triacetin, though a substrate of the adaptive ChE, failed to stimulate its production.

DISCUSSION

To further our understanding of the function of cholinesterases it would have been useful to discover a bacterial enzyme which was a necessary component of the cell metabolism under all conditions, possibly playing the same role in bacterial and animal cells. The bacterial enzyme here described, however, seems to be one which makes its contribution to the cell only when in a limited environment where an unusual nutrient must be made available. The enzyme is of interest because it is a new type of ChE and because the course of its adaptive formation is unusually slow.

Production of the enzyme

The enzyme is considered to be adaptive because the addition of ACh to the growth medium results in increased ChE activity of the culture. Certain other substances (choline and some factor in nutrient broth) also stimulate

enzyme production, but triacetin, a ChE substrate, does not. Since the increased activity of adapted cultures is seen in cell-free preparations, it cannot be simply explained on the basis of increased permeability to ACh.

The ChE appears in adapted cultures only at the end of a growth period of 2 or 3 days. Selection of ACh-utilizing mutants in the depleted medium might account for this, but such mutants should retain their high enzyme activity when subcultured into non-ACh media. We find, however, that a single subculture in tryptic digest broth suffices to abolish ChE activity almost completely. The delayed adaptation might be due to inhibition of enzyme production in the presence of substrates for the cell's constitutive enzymes, i.e. a 'diauxie' effect (Monod, 1947).

Properties of the enzyme

While the bacterial enzyme is in some respects similar to one or both major types of animal ChE, it is not identical with either (Table 6) (Augustinsson, 1948, 1949; Mendel & Hawkins, 1950).

Table 6. *Comparison of properties of animal and bacterial cholinesterases*

Enzyme Source	Nerve-type Nervous tissue Erythrocytes	Plasma-type Plasma Various organs	Bacterial <i>Ps. fluorescens</i>
Inhibition by prostigmine	+	+	+
Inhibition by excess of substrate	+	—	—
Rates of enzymic hydrolysis of choline esters:			
Fastest	Acetyl Propionyl Acetyl- β -methyl Butyryl	Butyryl Propionyl Acetyl Benzoyl	Propionyl Acetyl Acetyl- β -methyl (Butyryl)*
Slowest	(Benzoyl)*	(Acetyl- β -methyl)*	(Benzoyl)*

* Not split.

Both animal cholinesterases are inhibited by prostigmine at very low concentration, $<10^{-6}$ M. While concentration ranges were not explored here, the bacterial ChE was completely inhibited at 10^{-3} to 10^{-2} M., and partially inhibited at 10^{-6} M.

The effect of varying substrate concentration provides an important differentiating point between the two animal cholinesterases. The nerve enzyme shows the phenomenon of inhibition by excess of substrate, the activity rising to a maximum with increasing substrate and then decreasing at still higher concentration, to form a bell-shaped curve. The plasma enzyme shows a sigmoid curve, levelling off after a certain maximum enzyme activity is reached. The bacterial ChE does not show inhibition by excess of substrate over the range of concentrations where that phenomenon is seen with all known nerve type enzymes. Thus in this respect the bacterial enzyme resembles the plasma type.

Nerve and plasma cholinesterases have different substrate specificity patterns, and the bacterial enzyme is unlike either. The nerve type enzyme

splits ACh faster than other choline esters, and does not split benzoylcholine. The plasma enzyme attacks benzoylcholine but not acetyl- β -methylcholine, and splits both propionyl- and butyrylcholine faster than ACh. The bacterial enzyme clearly fits neither of these patterns. Like the nerve enzyme, it hydrolyses acetyl- β -methyl- but not benzoylcholine. It resembles the plasma enzyme in splitting PrCh faster than ACh, but differs in failing to split butyrylcholine. Thus substrates with more than two carbon atoms in the acyl group (butyryl- and benzoylcholine) are not attacked, while greater variation is tolerated in the alcohol portion (acetyl- β -methylcholine, Nu 2017-1, triacetin).

The bacteria appear to have three enzymes with lipase activity. Triacetin hydrolysis in powders is found only in association with the ChE (i.e. only in adapted powders). Its complete sensitivity to prostigmine, as well as the result of the mixed-substrate experiment show that the whole triacetinase activity of powders is due to ChE. Another enzyme must be present to account for the prostigmine-insensitive *triacetinase* activity of unadapted intact cells. This lipase is evidently destroyed by the acetone-drying procedure. Its effect on tributyrin is unknown. *Tributyrylase* activity is insensitive to prostigmine and is present in unadapted cells, hence cannot be ascribed to ChE. This lipase does not split triacetin, and its presence in powders further differentiates it from the triacetinase.

Part of this work was done during the tenure by one of us (A. G.) of a Lalor Fellowship.

Prostigmine, Nu 2017-1, propionyl- and butyrylcholine were kindly supplied by Hoffmann-LaRoche Inc., Nutley, N.J., U.S.A.

We wish to thank Dr J. Howard Mueller for the privilege of working in his laboratory.

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(Received 21 February 1952)

The Significance of Pleomorphism in *Mycobacterium tuberculosis* var. *hominis*

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SUMMARY: Under adverse conditions, *Mycobacterium tuberculosis* may autolyse, or surviving cells may divide into progressively shorter cells which may even appear to be cocal. We have not observed a complex 'life cycle', but the cells whose cytoplasm has been reduced show qualitative differences which we term 'regression'.

The significance of non-acid-fast forms, granules, beads, 'substance cyanophil' and spore-like bodies, observed in cultures of *Mycobacterium tuberculosis*, and in tubercular material, has been one of the major controversies of medical bacteriology during the past fifty years.

Our interest in this problem arose from frequent observation of acid-fast cocal forms in pus from tuberculous lesions of bone in which no bacilli could be found. We decided to investigate the morphological variability of human strains of *M. tuberculosis* under different conditions, and to see whether we could induce changes corresponding to any of the 'life cycles' previously described. Some of the conditions which we imposed on our cultures were chosen because they were said to be successful in inducing the L cycle of other bacteria.

MATERIALS AND METHODS

Phase-contrast microscopy. A Beck microscope with a 2 mm. objective and $\times 10$ eyepiece, and illuminated by a 6 V. Tenslite with an Ilford 404 green filter was used for intermittent observations, and two Cooke microscopes for the observation of microcultures over longer periods. The larger of these was described in this *Journal* (Lack, 1952). The other has a Newman-Waterfield warm chamber (Kaylene Ltd. London) which maintains a slide culture at 37°. Both these microscopes are fitted with Cooke phase-contrast systems and Ilford green filters.

Staining techniques. Ziehl-Neelsen, with and without counterstain, Much's and Gram's methods and 1% malachite green counterstained by 0.5% safranin. Klieneberger-Nobel's (1950) techniques of agar cut-out and osmic acid vapour fixation were used for cytological studies.

Microcultures. The majority were made on Dubos agar prepared by the addition of 1.5 g. New Zealand agar to 90 ml. Tween-Dubos medium, which was steamed for 1 hr., adjusted to pH 7.2, filtered, and autoclaved at 10 lb. pressure for 10 min. Bovine albumen solution was added at 55° to give a final concentration of 1/26.

Strains. H37Rv was used in most of this work, but a large number of strains has been freshly isolated from patients.

Culture media. Löwenstein, Dubos, and Bordet Gengou with 50 % human red cells, were used most frequently. In order that pleomorphism might be studied under various controlled conditions, the growth of H37Rv was also observed in the following media: Dubos, Dubos agar, glucose peptone water, glucose broth, 10 % glycerol in Dubos, yeast agar, 1 % urea in Dubos, 1.5 % glycine in Dubos. Cultures were made in urea-Dubos because granular forms were frequently observed in urine. Glycine was tried because it has been found to favour the production of L-forms in some other genera.

Strain H37Rv was also grown in the following biological fluids: serum from tuberculous and non-tuberculous patients; serum inactivated at 56° for 30 min.; pus digested with plasmin and deoxyribonuclease; synovial, pleural and amniotic fluids. The action of physical and chemical agents and of various antibiotics was also studied.

OBSERVATIONS

In eighty-eight experiments the growth of different strains was observed in a wide range of media. Only in media containing glycine or antibiotics did we observe the development of giant forms, and under no conditions did we observe a true life cycle in any way resembling that of fungi. Although the variations in pellicle production in liquid media were striking, and merit more attention, they were not studied further since they were not connected with morphological variation.

The Tables set out a summary of some of our observations representing the changes observed (i) in exhausted or deficient media (Table 1); (ii) when grown with different antibiotics (Table 2). These changes were observed mainly by means of phase contrast, often over long periods; if the photographs (Pls. 1-3) are studied before the Tables, terms such as typical bacilli (Pl. 2, fig. 5), autolysed rafts (Pl. 1, figs. 1, 2), degenerate bacilli (Pl. 1, fig. 3), short forms and 'diphtheroids' (Pl. 2, figs. 6*a*, *b*), and coccal forms (Pl. 1, fig. 4 and Pl. 2, fig. 7) will be better understood. Unless a stain is mentioned (z.n. = Ziehl-Neelsen, m.g.s. = malachite green safranin), the description is that of the phase-contrast preparation.

Multiplication. We saw each of the methods of multiplication of bacilli described by Hu (1936), and illustrated diagrammatically (Fig. 1*a*). Lateral budding was rare. Under some adverse conditions, surviving cells continued to divide in these ways, but as they did not grow so long, the daughter cells produced the coccal groupings that have been so frequently described (Fig. 1*b*). In very old cultures or under the action of some antibiotics, cells sometimes grew so little before they divided that they were already cocciform before they sent out a bud; these Hu described as 'sprouting granules'.

Autolysis. We watched the changes which occur when cells autolyse, and correlated the appearances seen by phase contrast with corresponding stained preparations. When individual cells were about to lyse, their nuclei became spherical and distended the cell wall (Pl. 1, fig. 3), giving the appearance of spores. When, on the other hand, the cells were in a cord or raft, autolysis began in one portion while cells elsewhere were still growing and dividing.

The outlines of the dead cells disappeared, the cytoplasm of adjacent cells fused and the nuclei became globular. The globules in this symplasm varied in size just as the nuclei varied in the cells before autolysis (Pl. 1, fig. 1). As the symplasm shrank and the nuclear globules were drawn closer together the mass had the appearance of a cluster of cocci (Pl. 1, fig. 2). These products of autolysis could persist for a very long time. Often they were embedded in

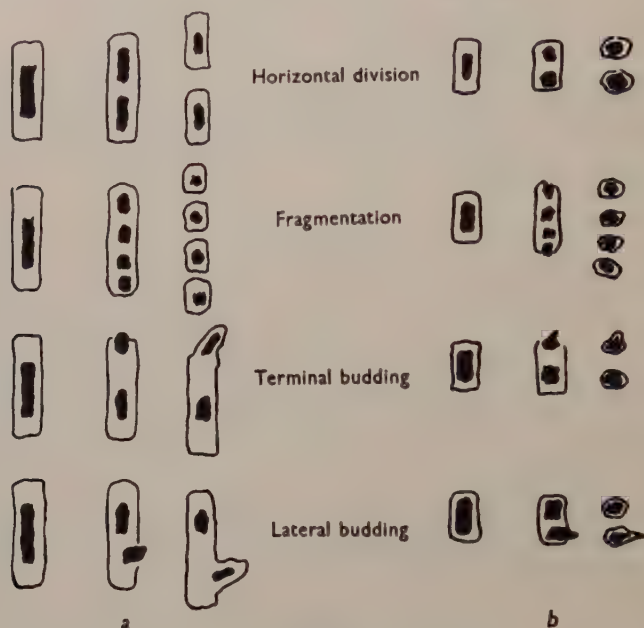


Fig. 1

long strands of extracellular material (slime, 'substance cyanophil'). If bacilli were still dividing close to an area of autolysis, they were likely, for reasons we give below, to be short or even coccal in form, so that it might appear as though they had their origin from this symplasm. We never observed regeneration from cells which had undergone autolysis, either individually or *en masse*.

Though subcultures and observation of growth in microcultures were used to check viability, we found during the course of these observations that the malachite green safranin stain was quite a reliable indication of the state of a culture—viable bacilli staining green and dead or dying bacilli staining pink, though both might have stained red with the orthodox Ziehl-Neelsen stain. In this connexion we confirmed Nedelkovitch's observation that uninoculated egg media may contain acid-fast material which, after staining with Ziehl-Neelsen, can easily be mistaken for the smaller forms of *M. tuberculosis* (Nedelkovitch, 1950).

The biological fluids listed all appeared to retard growth with the exception of digested pus. Treatment of tuberculous pus with 'streptokinase-strepto-

Table 1. *Changes observed in H 37 Rv during prolonged culture under varying conditions*

Dubos + Tween 80 + albumin. 37° for 7 weeks. Room temp. in dark for 12 months	Dubos + Tween 80 + albumin. 37° in dark for 12 months	0.1 % glucose broth at 37° in dark	10 % glycerol broth at 37° in the dark	Dubos + 1.5 % glycine at 37°
Cords of bacilli. Acid-fast with z.n. Mostly green but a few red with m.g.s. Show very little change in morphology after 12 months at room temperature.	After 4 months, rafts of bacilli showing areas of autolysis as in Pl. 1, fig. 1. After 5 months, remnants of autolysed rafts, numerous short forms. z.n.: swollen bacilli, acid-fast cocci. m.g.s.: red bacilli short green forms. After 12 months, similar appearance. <i>Subcultured</i> into Dubos and digest broth gave a growth of typical acid-fast bacilli in 3 weeks.	At 1 week, bacilli, short rods and cocci. z.n.: acid-fast bacilli, 'diphtheroids' and cocci. At 2 weeks, fewer bacilli, more short forms. At 1 month, rafts of bacilli now numerous. z.n.: numerous acid-fast bacilli. <i>Subcultured</i> on to Lowenstein's medium. Growth in 2 weeks.	At 6 weeks, bacilli growing in ropes. z.n.: acid-fast bacilli with some short forms. At 14 weeks, ropes of bacilli have now autolysed. Numerous short forms. Now kept at room temperature in dark for 6 months—only short forms remain. <i>Subcultured</i> into Dubos and digest broth. Growth of typical acid-fast bacilli in 6 weeks.	1st day, typical bacilli. 4th day, club and filamentous forms. 7th day, some autolysis. 10th day, photographed Pl. 3, fig. 10. 16th day, bacilli shaped like pearls, drum sticks and parsnips. 25th day, tadpole forms still acid-fast. <i>Subcultured</i> into Dubos without glycine. Short bacilli appeared in subculture on 4th day and by 19th day had become numerous.
COMMENTS	COMMENTS	COMMENTS	COMMENTS	COMMENTS
The rate of growth having been retarded by lowering the temperature, nutrition remained adequate for 12 months.	Continuous culture at 37° has resulted in limitation of cell size by nutrients. The viable (green) short forms grow to normal size after subculture.	There was a rapid autolysis of bacillary forms, after which short forms lengthened. Medium may be temporarily enriched by autolytic products.	Limitation of cell size by nutrients with restoration to normal size after subculture.	Some interference with cell division by glycine.
	z.n. = Ziehl-Neelsen.		m.g.s. = Malachite green safranin.	

Table 2. *Observations of H 37 Rv exposed to various inhibitory substances*

Dubos + streptomycin 0.6 μ g./ml.		Dubos + streptomycin 0.6 μ g./ml. + <i>p</i> -amino- salicylic acid 0.6 g. % (w/v)		1 % glucose peptone water + 20 units penicillin	
1st day: typical bacilli, some in rafts.		1st day: typical bacilli some in rafts.		1st day: normal bacilli.	1st day: clumps of bacilli.
4th day: pleomorphic bacilli, some giants, some very short. Occasional club-forms.		4th day: autolysis.		4th day: irregular forms, some long with terminal swellings.	4th day: no change.
7th day: autolysis of rafts, pleomorphic bacilli, some loss of acid-fastness.		7th day: autolysis. Short forms.		9th day: long beaded bacilli, 'diphtheroid' and coccal forms.	9th day: no change.
11th day: short forms losing acid-fastness. Numerous short forms (Pl. 3, fig. 8).		11th day: short forms losing acid-fastness. Numerous short forms (Pl. 3, fig. 8).		19th day: numerous 'diphtheroid' and coccal forms.	2 months: still numerous cords of bacilli, some pleomorphism.
15th day: bacillary forms have nearly disappeared.		15th day: bacillary forms have nearly disappeared.		23rd day: numerous acid-fast short forms (Pl. 2, fig. 7).	<i>Subcultured</i> on to Löwenstein produced heavy growth.
23rd day: debris of autolysis. Numerous granules.		23rd day: debris of autolysis. Numerous granules.		M.G.S.: numerous green short forms and debris. <i>Subcultured</i> after 6 months;	
Occasional acid-fast 'diphtheroid' and coccal forms.		Occasional acid-fast 'diphtheroid' and coccal forms.		Dubos good growth after 3 weeks. Digest broth scanty growth after 8 weeks.	
35th day: no change.		35th day: no change.			

COMMENT ON ACTION OF STREPTOMYCIN AND *p*-A.S.A.

A number of cultures were observed, with varying concentrations of these substances, both singly and in combination. The results were so variable that presumably drug-resistant mutants were complicating the studies at lower concentrations. In the three reported here, no growth was obtained on subculture on the 35th day. In another culture of the same strain in Dubos with 0.8 μ g. streptomycin/ml., some 'green' bacilli persisted throughout, and subculture on to Löwenstein's medium at the end of six weeks produced growth. Streptomycin may produce giant forms; both growth inhibitors produce autolysis and regression to a varying degree.

COMMENT ON GLUCOSE PEPTONE WATER AND PENICILLIN

These observations are recorded because the presence of penicillin, by comparison with the control glucose peptone culture, appears to have accelerated regression.

dornase' (Burroughs Wellcome and Co.) enhanced the growth of tubercle bacilli and permitted degraded forms to grow on Löwenstein medium that otherwise would not (Tanner, 1951).

We have begun the study of morphological changes that occur immediately after inoculation into animals. A fuller account of this will be reported later, but attention may be drawn to the photograph (Pl. 3, fig. 9) of the 'diphtheroid' type of bacilli recovered from a rabbit's peritoneal exudate 10 days after intraperitoneal inoculation of a suspension of living bacilli. The strain H37Rv was inoculated intraperitoneally into rabbits, guinea-pigs and rats, and their peritoneal exudates were examined at intervals. No fully developed acid-fast bacilli could be found in the deposits of these exudates in the early stages, but numerous short acid-fast rods and cocci, both free and in macrophages, were seen, and culture on Löwenstein gave a luxurious growth of tubercle bacilli.

Lastly, our findings are illustrated in one of the number of clinical infections which provoked this study. A young man noticed a painless swelling on his right knee following exercise. This was treated by diathermy for 2 months without benefit. After 3 months a biopsy of the synovial membrane of this joint was taken. Histological examination showed no inflammatory reaction and no evidence of tuberculosis. Staining by the Ziehl-Neelsen method showed cocco-diphtheroid forms in the synovial membrane and fluid (Pl. 3, fig. 11), and inoculation of this material into a guinea-pig produced tuberculosis with typical acid-fast bacilli in the guinea-pig spleen (Pl. 3, fig. 12).

DISCUSSION

The two conclusions that we have reached from these studies are (a) that the process and appearance of autolysis has confused many workers in the past and discounts much of what has been written about stages in a 'life cycle', and (b) that the progressive shortening of bacilli under some adverse conditions, a process which we propose to call 'regression' instead of the usual term 'degeneration', represents not merely a reduction in size of the organisms but qualitative changes that have important implications.

Grigoraki (1950) seems to have confused these two processes. In his account of the sexual phase of development, the ectoplasm of lysed bacilli fused to form an amorphous veil in which the nuclei were transformed into granules which fused to form a symplasm. He describes stalks growing out from the symplasm bearing large ovoid cells which on bursting release male and female cocciform gametes. Short rods are supposed to form by fusion of these gametes. It seems more likely that the fusion and lysis of cells and the release of the nuclei therefrom is a process of autolysis, and that the short rods are derived from bacilli which have escaped autolysis, though they may still be attached to, and appear to grow out from, a clump of bacilli which have lysed.

Pl. 1, figs. 1 and 2, illustrate how easily such a confusion may arise, particularly if the preparation had been fixed and stained instead of being observed by phase contrast. Pl. 1, fig. 1, shows the process of autolysis in a raft of bacilli. Autolysis is spreading from right to left in this picture but

'coccal' and 'diphtheroid' forms derived from dividing bacilli are appearing in the top left-hand quadrant.

When the symplasm of an autolysing raft has disappeared and the nuclei are clumped together, as we see in Pl. 1, fig. 2, the multiplication of a few surviving bacilli may well appear to result from the fusion of 'coccal forms'.

Schaefer, Marshak & Burkhart state that in the absence of antibiotics, exhaustion of the nitrogen source in the culture medium is an essential condition for the appearance of autolysis, and that both oxygen and a source of energy such as glucose are necessary (Schaefer, Marshak & Burkhart, 1949; Marshak & Schaefer, 1952). In the presence of streptomycin, penicillin and *p*-amino salicylic acid, autolysis always occurs; giant forms and monstrosities appear in the presence of streptomycin and of glycine.

The important issue in this discussion is the significance of the coccal forms. We wish to emphasize that there may be three kinds of 'coccal' forms in a stained preparation: (a) nuclear remnants from autolysed bacilli appear 'coccal'; (b) in stained preparations coccal forms may be produced by the detachment of growing tips of bacilli in the smearing of cultures (these may frequently be observed as round bodies close to the growing end of mature bacilli, and may be regarded as artefacts—Yegian & Porter, 1944); (c) the coccal forms which are of the greatest interest are those which are produced in large numbers under adverse conditions by the division of diphtheroid forms, and which stain green with the M.G.S. stain.

Bisset's (1949) view is that tubercle bacilli are multicellular, a single bacillus containing up to twelve or more cells. We agree that this is so when it is fragmenting, but in our studies of human strains this mode of multiplication is uncommon. Growing from the tip of a bacillus with subsequent snapping of the newly formed bacillus to a Λ is seen most frequently among isolated bacilli, whereas in clumps the added bacilli form cords. Under adverse conditions, fragmentation, transverse division and lateral budding have all been observed, and as the bacilli become progressively shorter, multiplication in these ways gives rise to 'coccal' groupings.

A statement that cells diminish in size as they divide in an inadequate medium may appear so trite as to merit no further consideration. That the reduction in nitrogen content may be very considerable is shown by the findings of Marshak & Schaefer (1952) who report that 'in the presence of adequate amounts of glucose in a medium deficient in a source of nitrogen, tubercle bacilli continue to proliferate', and that 'the cells of the second generation (fourth day) produced under these conditions have a nitrogen content per milligram of dry weight which is only approximately 40 % of that in the cells used as inoculum. Relatively little of the cellular nitrogen originally added is lost to the medium.' The weight of the culture was nearly doubled and the final density was 2.6 times the initial density. They did not report their observations on the morphology of these organisms, and their statement as it stands might be taken to imply a 2½-fold relative increase in non-nitrogenous cell constituents. However, we find a marked reduction in size of dividing cells not only with nitrogen starvation but also in the presence

of antibiotics and biological fluids, and from our few initial observations it appears that bacilli dividing inside macrophages also tend to become diphtheroid or coccal. These coccal forms are capable of growing into full-bodied bacilli when transferred to a suitable environment.

Vaudremer (1926) showed that tubercle bacilli, when grown on poor media, become non-acid-fast and pleomorphic and cease to produce tuberculin. On return to suitable media they recovered their acid-fastness. Pagel (1934) found in histological sections of tuberculous foci slender chromophilic bacilli in some lesions and short plump chromophobic bacilli or granules in others. The former produced more rapid development and larger tuberculous infiltrations in guinea-pigs than the latter. We also found a striking difference in the morphology of bacilli from tuberculous sinuses where the bacilli are large and well formed, and those from closed lesions in bone where the organisms are usually coccal. The former produced rapid development of tuberculosis in guinea-pigs, the latter slowly or not at all; since the use of streptomycin, organisms seen in tuberculosis pus from sinuses are now usually like those from bone, that is, short, poorly acid-fast and with low infectivity.

Brieger, Miles, Cosslett & Horne (1951) recovered granular forms of bovine tubercle from the spleens and lungs of infected rabbits and found these to be infective to guinea-pigs, though they would not grow on laboratory media. Kahn & Nonidez (1936) found that culture filtrates of H37 and of a bovine strain that had been through membrane filters of a pore volume from 0.5 to 0.1 μ caused tuberculosis in four out of five guinea-pigs, though inoculations on to Petroff's, Long's, Löwenstein's and glycerol agar medium were sterile. We have also encountered the anomaly of granular forms in infected material which would not grow on routine media but which infected guinea-pigs, though in this respect growth has occurred on Bordet Gengou's medium with 50 % human red cells added, when the other media have remained sterile.

We suggest that tubercle bacilli, carried by the blood from a primary or secondary focus, may lodge in areas in the skeletal system and in other sites in which the micro-environment is unfavourable for full development, and that there the organisms may regress and remain dormant until the environment is altered in their favour by injury, haemorrhage, metabolic changes or infection by other organisms.

We have found short diphtheroid and coccal forms staining green with the malachite-green safranin in which no bacilli or tubercles could be found in joint tissue biopsies, and this tissue has infected guinea-pigs with tuberculosis. The patients have invariably been tuberculin-positive (evidence of an antecedent infection), but showed no signs of an active tuberculosis until after trauma. In our view, this suggests that the organisms were in the joint tissues in a form that did not produce a characteristic reaction even in sensitized hosts until the change in their environment allowed them to grow into bacilli again. Once this has occurred the characteristic tissue reaction follows.

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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. Autolysis within a raft of bacilli. H37Rv. 3-week Dubos culture. Phase contrast. $\times 4000$.
- Fig. 2. Autolysis at a later stage: the nuclei resemble a cluster of cocci. H37Rv in Dubos with streptomycin and *p*-A.S.A. Phase contrast. $\times 4000$.
- Fig. 3. H37Rv in Dubos. 2 months. Bacilli about to lyse.
- Fig. 4. H37Rv. 1 year in glucose peptone water—still viable. Short forms resembling cocci. Much's stain. $\times 2000$.

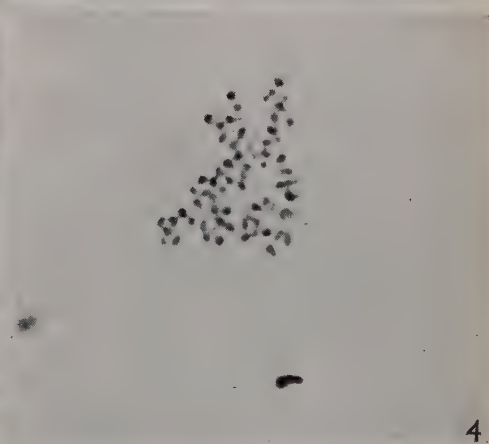
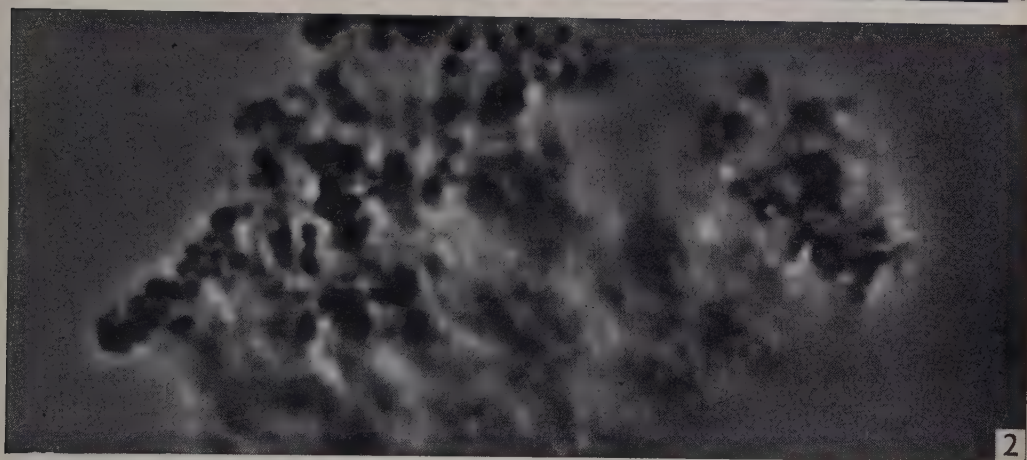
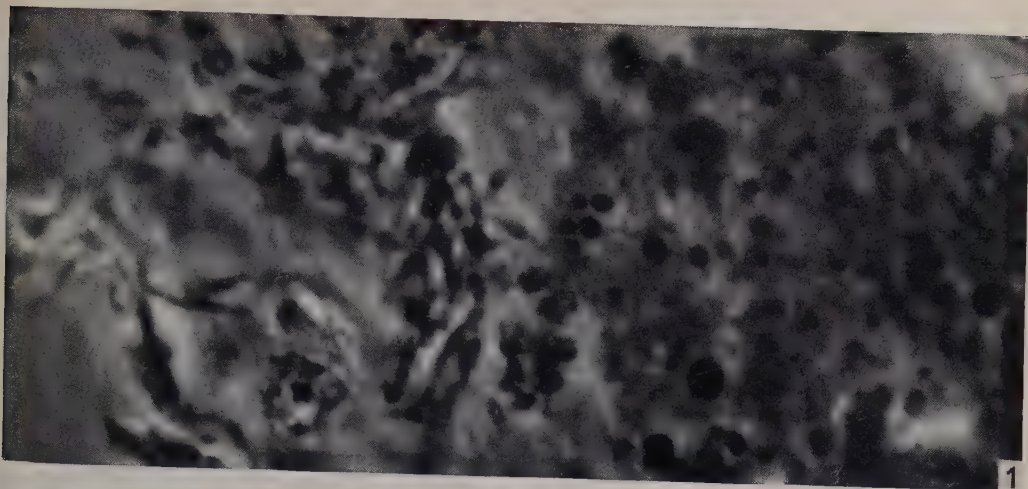
PLATE 2

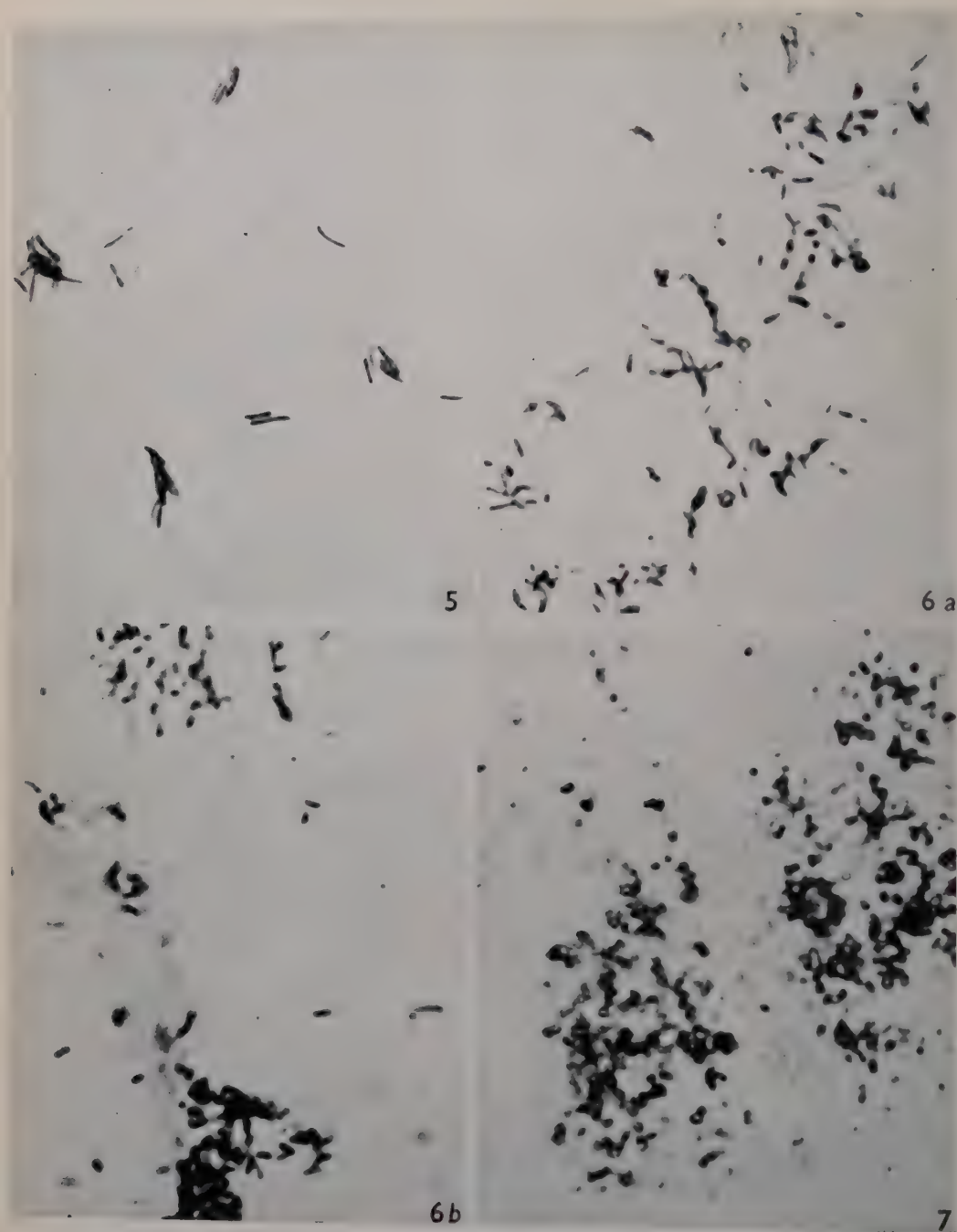
- Fig. 5. H37Rv. 14-day Dubos culture—normal bacilli. z.N. stain. $\times 2000$.
- Fig. 6a, b. H37Rv. 15-day culture in Dubos plus 0.17 μ g. streptomycin/ml. Short forms predominate. z.N. stain. $\times 2000$.
- Fig. 7. H37Rv. 19-day culture in glucose peptone water plus 20 units penicillin/ml. Short forms and autolysis. z.N. stain. $\times 2000$.

PLATE 3

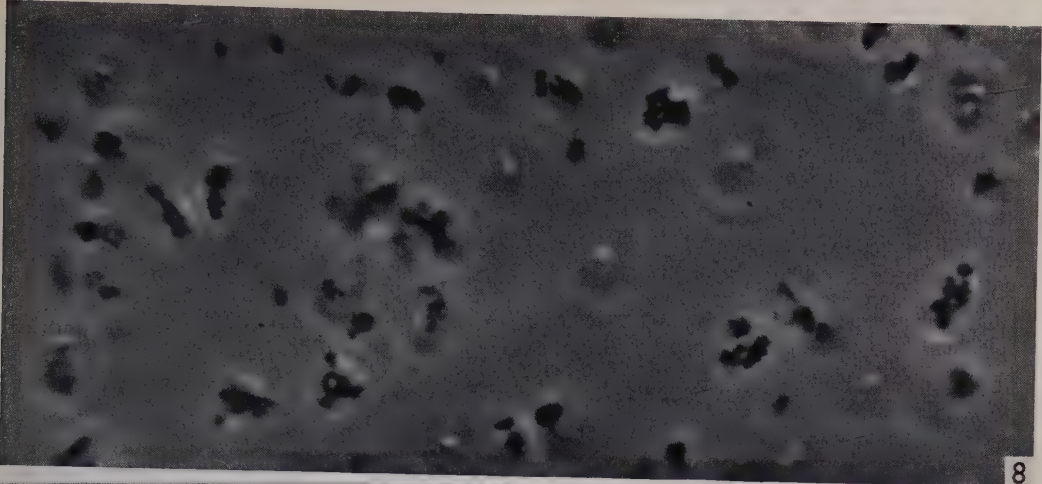
- Fig. 8. Short forms of H37Rv grown in Dubos with *p*-A.S.A. Phase contrast. $\times 4000$.
- Fig. 9. Short forms of H37Rv recovered from rabbit peritoneal exudate 10 days after intraperitoneal inoculation. Phase contrast. $\times 4000$.
- Fig. 10. H37Rv in Dubos plus 1.5% glycine for 10 days. Abnormal nuclei. z.N. $\times 2000$.
- Fig. 11. Acid-fast cocco-diphtheroid forms in the synovial fluid of a patient in which no acid-fast bacilli were found. z.N. stain. $\times 3000$. Ilford micro 2 filter has been used to reduce the density of the surrounding cells.
- Fig. 12. Acid-fast bacilli in the spleen of a guinea pig injected with the fluid illustrated in Fig. 11. z.N. stain. $\times 3000$.

(Received 3 March 1952)

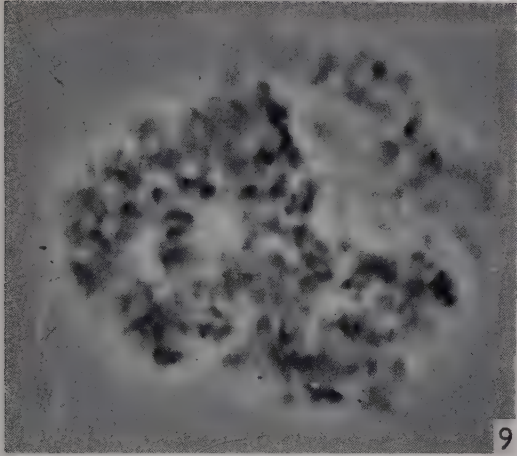




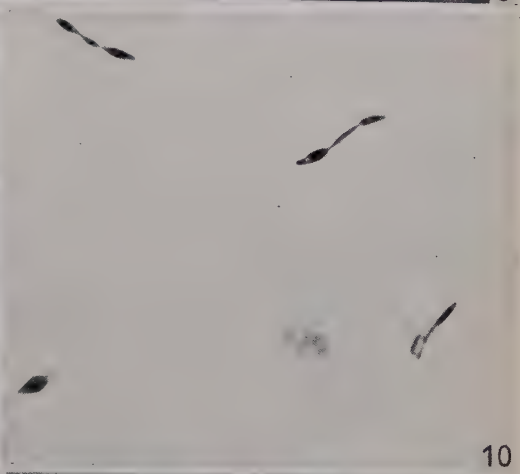
C. H. LACK & F. TANNER—PLEOMORPHISM OF *M. TUBERCULOSIS*. PLATE 2



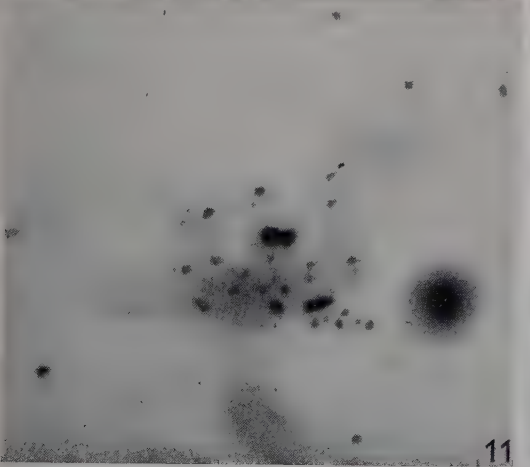
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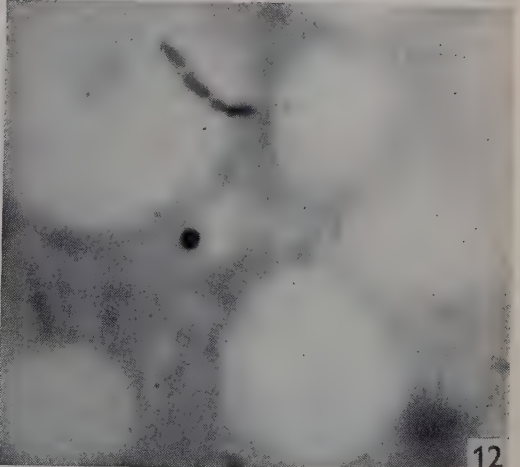
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The Heat Resistance of *Streptococcus faecalis*

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SUMMARY: The log survivor/time graph for mature cultures of one strain of *Streptococcus faecalis* was not always straight over its whole length. A preliminary period of slow death was frequently observed, and a final similar period less often. The log survivor/time graphs of young cultures varied in shape with age of culture and often showed an initial rapid death-rate, passing suddenly into a slower one.

The heat resistance of three strains was considerably influenced by the age of the culture. Resistance, judged either by the Decimal Reduction Time or the time taken to reach 99.9% mortality, was increased by transfer to fresh medium and fell during the lag phase of growth, reaching a minimum as rapid reproduction began. A second maximum in heat resistance occurred when growth became slow: thereafter the resistance gradually fell to that of mature cultures. The incubation temperature affected the heat resistance of young cultures, the effect of high temperatures being particularly marked and resulting in increased resistance, except with cultures 1 hr. old or less.

The heat resistance of the faecal streptococci has been the subject of much investigation and discussion ever since Houston & McCloy (1916) laid such stress on the ability of their strain to withstand heat. Nearly all measurements of resistance have been by 'end-point' methods, which are open to criticism because they place undue emphasis on a few highly resistant cells which are not typical of the population as a whole. A study of the death-rate of the majority of the cells in a culture should provide a better indication of thermal resistance.

Richards & White (1949) showed that the resistance of *Streptococcus faecalis* to moist heat at 60° may be conveniently expressed by the Decimal Reduction Time (DRT) of Katzin, Sandholzer & Strong (1943) which is obtained from the relationship between the logarithms of the survivors and time. Fairly consistent DRT values of approximately 1 min. were obtained with various strains, but the death-rate was not always constant. The present paper presents the results of further investigations on these lines.

METHODS AND MATERIALS

General technique. The technique used throughout this work was that described by Richards & White (1949). Briefly, a standardized number of cells was added to a flask of saline held in a water-bath at 60° and the survivors determined in samples taken from this flask at timed intervals. The fixed number of cells was obtained by centrifuging glucose Yeastrel broth cultures and resuspending the deposit in saline to a standard turbidity. Counts of survivors were obtained by the plating method using glucose Yeastrel agar, two or three plates being made of each dilution.

Cultures. The strains of *Strep. faecalis* used were isolated, either from sour milk, human faeces or canine faeces, by the author. Six authentic strains kindly supplied by Dr P. M. F. Shattock were also used. Three strains were studied most, one (L5) from human faeces, one (L6) from canine faeces; the third strain (C and G), from milk powder, was supplied by Dr Shattock. The cultures were characterized by morphological and biochemical tests, and their identity confirmed by these tests at intervals. They were maintained in cooked meat medium, in which they were transferred every 3 months. Cultures for heat resistance tests were grown in glucose Yeastrel broth (1% peptone, 0.25% sodium chloride, 0.3% Yeastrel, 1% glucose, pH 7.2) under the required conditions.

Young cultures. Very young cultures needed to be inoculated into the broth more heavily than usual to give, during the short incubation, sufficient cells for an experiment. Various trials were made to find the lowest possible inoculum, it being clearly desirable to keep this small to ensure that the number of older cells in the standard suspension should be as low as possible. Eventually 0.1 ml. portions of parent (18 hr.) cultures were inoculated into glucose Yeastrel broth: when necessary the deposits from several tubes were combined.

Incubation. To ensure accuracy in timing short periods of incubation, a water-bath controlled to $\pm 0.5^\circ$ was used and the broth tubes brought to the proper temperature in the bath before inoculation.

When the effect of alteration of incubation temperature was being studied, the question of the temperature at which to incubate the parent culture arose. Ellicker & Frazier (1938) found that the incubation temperature of the parent cultures greatly influenced the resistance of *Bacterium coli*, especially during the lag phase of growth, in which 28° cultures inoculated from a 38.5° parent culture showed greater resistance than did 38.5° cultures inoculated from parents at either 38.5 or 28° . It was finally decided to incubate the parent culture at its optimum (37°) at this stage of the work so that effects due to varying incubation temperature would have been produced during one subculture only.

Direct microscopic counts. Two separate wire loopfuls were removed vertically from the incubating broth culture and spread over separate 1 sq.cm. areas on different slides. The smears were then dried quickly, fixed and stained. The average number of units in a microscope field was then obtained by counting five to ten fields on each slide, groups and individual cells being each treated as units. It was noted that the size of the groups varied greatly with the age of the culture, but the results from the two slides generally agreed well.

Jennison (1937) with *Bact. coli* concluded that, as the amount of clumping varied, the plate count could not bear a constant relationship to the total count. Obviously, the relationship must be far more variable with chain-forming organisms such as streptococci, but counting both groups and individual cells as units should reduce the discrepancy between the results of the two methods, and the direct count had the advantages of speed and economy in materials.

Estimation of resistance. The Decimal Reduction Time (DRT) was obtained from graphs on which the logarithms of the survivors were plotted against time. The steepest straight part of the line was always used for calculation purposes, so any preliminary periods of slow death or any tailing-off periods towards the end were not allowed for in the DRT values. It was realized that values so obtained would not take into account the characteristic features of some curves but their use was felt to be justified for the purposes of comparison.

To give a more complete picture of resistance in cases where the logarithmic death-rate varied during an experiment, 99.9% mortality times were also obtained. These were read off from the log survivor/time graphs, using the count of the first sample as the reference value. Since the experiments were originally designed to yield information about variations in DRT only, it had not been envisaged that counts of the numbers of organisms added to the saline in the flask would be required.

Assessment of initial and final periods of slower death. The steepest straight portion of the log survivors/time graph was extrapolated backwards, and the time at which this line reached the level of the count in the first sample (usually about $\log=7$) was taken as the duration of the initial period of slow death. The final period of slow death was assumed to begin where the experimental data indicated a break from the portion of steepest slope.

RESULTS

The shape of the death-rate curve

It has already been shown that the log survivor/time relationship for *Strep. faecalis* exposed to heat at 60° is not always linear (Richards & White, 1949). In many cases an initial portion of small slope is observed, the slope increasing gradually to a maximum which is usually maintained until the end of the test, though there may be a resistant tail of organisms whose death-rate is slower. Preliminary periods of slow death are important and must be borne in mind when comparing the heat resistance of various organisms. Some means of indicating its occurrence and duration should be included in any expression of heat resistance, and its frequent appearance with *Strep. faecalis* strains seemed to merit more detailed study.

Mature cultures. A preliminary survey was made of twenty experiments which were selected because they were all done within a few days on mature cultures of strain L5 incubated at 37°. Records of the incidence and duration of initial periods of slow death and the time of onset of tail periods showed that in three cases there was no initial period of slow death at all, fourteen had slow periods up to 2 min. and three slow periods up to 3 min. There was no tail in fourteen cases, a tail from 4 min. in five cases and a tail from 6 min. in one case. From this it was concluded that while an initial period of slow death of just over 2 min. was usually present, a tail period occurred less often, and when present did not appear until at least 4 min. after the beginning of the experiment.

Length of period of slow death in mature cultures. As all the above experiments were carried out with samples taken at the end of the first minute after subjection to heating and at 1 min. intervals thereafter, it was realized that more exact information on the duration of the period might be obtained by staggering the sampling times in a series of experiments. The sampling was still at 1 min. intervals but the time of taking the first sample was varied. Twelve successive experiments of this type on strain L5 gave a mean duration for the initial period of slow death as 2.25 min.

A survey of the incidence and length of this period of slow death with other strains of *Strep. faecalis* exposed to heat at 60° was also made, and it was concluded that, although not always present, it occurs frequently. It was almost invariably shown by strains L5 and 61, and also by the six strains supplied by Dr Shattock. Strains L6 and 62 showed it sometimes and strain 63 hardly ever. Strain 64 was abnormal, the death-rate being very slow: although followed for 25–30 min., no change in rate could be detected.

Young cultures less than 12 hr. old. These gave very variable results and the exact age of the culture was important. Fig. 1 shows log survivor/time graphs for strain L5 at 37°. The culture 30 min. old was highly but uniformly resistant while the 2.5 hr. culture showed an initial fast death-rate which changed suddenly to a lower value. Fig. 2 illustrates the behaviour of strain L6. Cultures 1 hr. old showed an initial period of slower death followed by one of constant faster death, while the 1.5 hr. culture showed an initial rush and sharp break. With the other strains the break was often less sudden and the graph approximated more to a curve of gradually lessening slope. Thinking that the variability might have been due to heterogeneity in the young cultures, i.e. that cells which had not yet succeeded in dividing were mixed with those in active division, an attempt was made to make the population more uniform by subculturing serially after incubation for 2 hr. Fig. 3 shows the result using strain L5, with organisms from the fourth subculture at 37°. The death-rate is uniform.

The influence of age of culture on DRT

Strain L5; 12–48 hr. cultures. Fifty-two experiments with 22 hr. cultures of strain L5 gave a mean DRT of 1.02 min. The values from cultures 12, 18, 36 or 48 hr. old (shown in Table 1) were not significantly different from this. This finding may be compared with that of Stark & Stark (1929) who observed that the resistance of *Strep. faecalis* cultures increased up to 24 hr. after which it decreased up to 48 hr.

Strain L5; cultures less than 12 hr. old. Replicate experiments (usually five or six, never less than three) were carried out on cultures 0.5–8 hr. old. The DRT's varied somewhat from experiment to experiment, but Fig. 4 shows how the mean value of the DRT varied with the age of the culture within this period. It may be seen that the youngest cultures (30 min. old) were much more resistant than the parents, having a mean DRT of 3.1 min. against about 1 min. for the parent cultures. There was a further slight rise in resistance for the next 30 min. of incubation, after which a steep decline set in, a minimum

value being reached at about 2 hr. This was followed by a gradual rise to a second but less high peak after about 5.5 hr. Later there was a gradual fall in resistance to the figure of about 1 min. at 8 hr., and the figure was still approximately the same at 12, 18, 36 and 48 hr. as already shown.

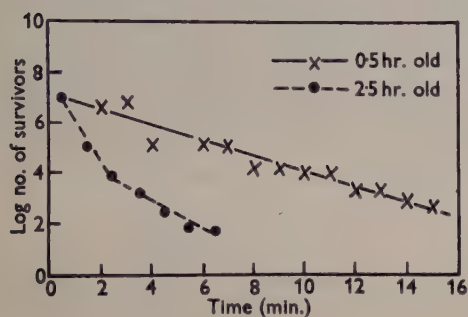


Fig. 1

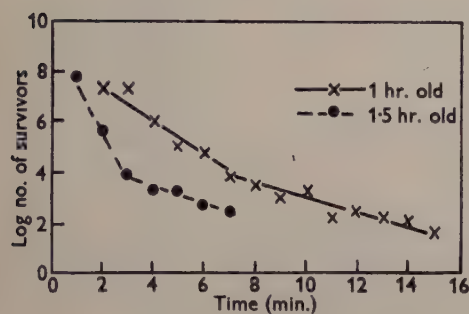


Fig. 2

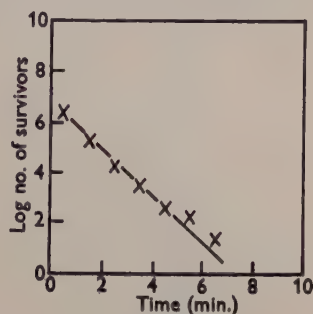


Fig. 3

Fig. 1. The log survivor/time graphs for typical young cultures of *Strep. faecalis* strain L5 exposed to heat in saline at 60° (incubation at 37°).

Fig. 2. The log survivor/time graphs for typical young cultures of *Strep. faecalis* strain L6 exposed to heat in saline at 60° (incubation at 37°).

Fig. 3. The log survivor/time graph of a 'built-up' young culture (four successive 2 hr. subcultures) of *Strep. faecalis* (strain 'L5') at 60° in saline.

Table 1. *Effect of age of culture on resistance of Strep. faecalis (strain L5) incubated at 37°*

	Age (hr.)			
	12	18	36	48
	DRT (min.)			
	1.25	0.91	0.91	1.11
	1.18	1.0	0.69	1.0
	1.11	1.43	0.5	
	1.05	0.95	0.91	
	0.67		1.0	
	0.67		0.8	
	0.91			
Mean	0.98	1.07	0.8	1.06

Results with other strains. Young cultures of strain L6 and strain C and G gave resistance curves of the same general shape as that for L5, though they varied in detail. With strain L6, the first stage of greater resistance lasted longer and the point of least resistance was delayed until about 3 hr. The second peak of resistance occurred between 6 and 9 hr., i.e. the whole cycle of resistance took longer to accomplish. With strain C and G the first stage of increased resistance was shorter than with L5, and after only 1 hr. of incubation the point of minimum resistance was reached. The second peak was also reached slightly sooner, in about 4.5 hr.

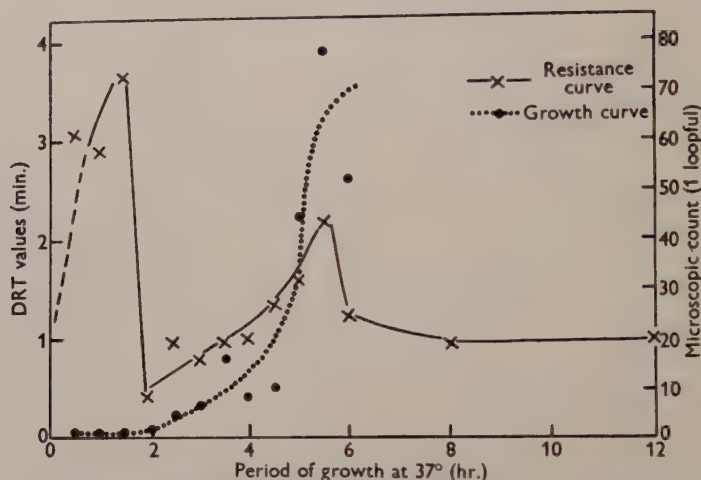


Fig. 4. The effect of the age of the culture on the resistance (expressed as DRT) of *Strep. faecalis* strain L5 at 60° in saline (incubation at 37°).

The relation between resistance and growth phase. To see whether this variation in timing but not in general shape of the resistance curves was associated with differences in the growth-rates of the different strains, the development of broth cultures was followed by means of direct microscopic counts. Several experiments were carried out and the average counts plotted against time of incubation. The growth curve for strain L5 is shown in Fig. 4 together with the resistance curve.

There was a marked association between the phases of the growth curves (inaccurate as they were) and those of the resistance curves, especially with strain L5. It will be seen that during the lag period of growth this organism showed its greatest resistance to heat. As soon as active reproduction commenced, or possibly slightly before, resistance fell to the minimum. As growth proceeded through the logarithmic phase resistance rose again and may have reached the second peak at the end of that phase, but counts were not carried on sufficiently long for this to be established definitely.

One of the more interesting features of these age/resistance curves is the spectacular rise in resistance of the very young (lag phase) cultures. Within a short time of subculturing to fresh medium (as little as 15 min. with some

strains) the resistance had at least doubled. Further investigations into this point are planned.

With all three strains it was noted that cultures up to 4 hr. old had more large groups and chains than the older cultures. Probably a temporary increase in chain length is an indication of rapid multiplication. For this reason, the microscopic counts cannot be said truly to portray the increase in cell numbers with time; but the same criticism would apply to plate counts had they been done instead. However, the observation of this increase in chain length is of some importance as it may explain to some extent the less regular death-rates of these young cultures.

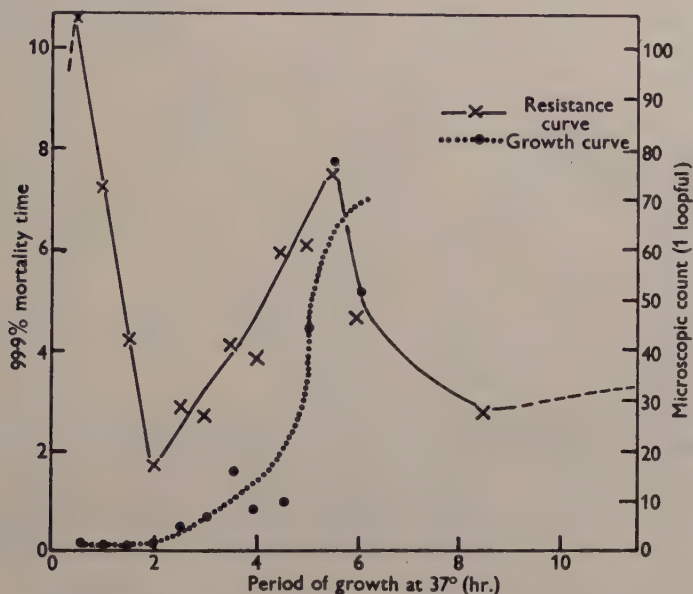


Fig. 5. The effect of the age of the culture on the resistance (expressed as 99.9 % mortality time) of *Strep. faecalis* strain L5 at 60° in saline (incubation at 37°).

The influence of age on mortality time

It is evident that when resistance is estimated in terms of the DRT which is calculated from the steepest portion of the log survivor/time curve, the estimates err by not taking into consideration the length of any preliminary period of slow death. This is especially important with very young cultures, where the logarithmic death-rate is by no means constant. Accordingly, 99.9 % mortality times were obtained from the same curves. Naturally, variations in the shapes of different curves influenced the results considerably, but the general relationship between age and resistance was not altered. This may be seen from Fig. 5 where the results for strain L5 are shown. Similar results were also given by strains L6 and C9, and it may be concluded that the resistance of young cultures of *Strep. faecalis* does indeed vary in a constant manner according to the growth phase.

*The influence of incubation temperature on the
resistance of young cultures*

Strain L5. The results of experiments carried out with young cultures of strain L5 incubated at 27° are shown in Fig. 6. The general pattern of changes in resistance was the same as at 37°, and there was clearly the same association with the growth phases. The lag phase was longer (about 4 hr. instead of 2 hr. as at 37°) and resistance fell steadily as it progressed, reaching a minimum

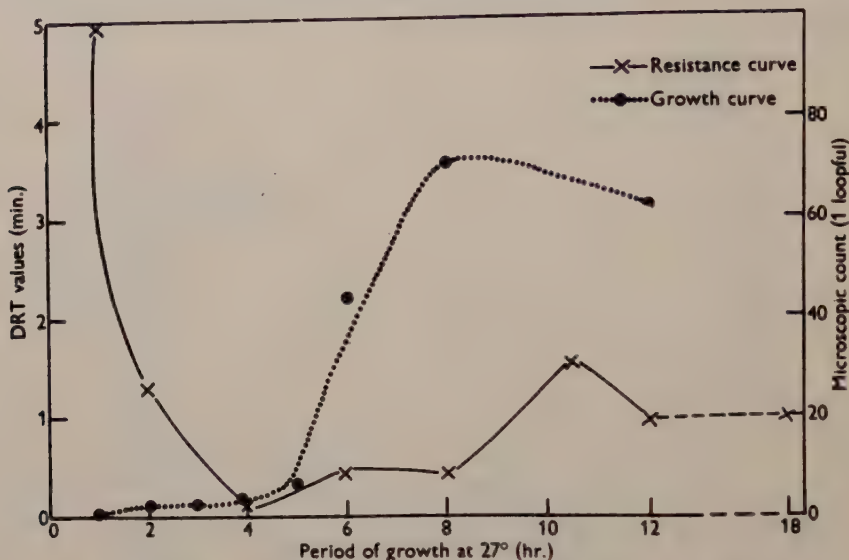


Fig. 6. The growth curve and resistance curve of young cultures of *Strep. faecalis* strain L5 incubated at 27°.

just as active growth began. The whole cycle of changes took far longer at 27° than at 37°, due to the slower growth rate. However, the difference between the maximum and minimum resistance was much greater. During the lag phase there was a higher DRT than at 37°, but when the resistance dropped, as the logarithmic phase began, it fell much below the value for 37°. It is possible that the apparently exaggerated changes in the resistance at 27° came about, in part, because it was easier to obtain truly representative cultures of each age when the rate of growth was slower. After the minimum value the DRT rose slowly to a minor peak in 10 hr. cultures and subsequently declined to about 1 min., at which level it remained constant. The final condition was thus the same as in mature cultures at 37°.

At 45° also, the general shape of the resistance curve was the same as at 37° (Fig. 7), but the point of minimum resistance was reached at about 5 hr. The direct microscopic counts of the 45° cultures were not very satisfactory, since the organisms tended to form into large clumps and tangled chains which were sometimes clearly visible to the naked eye as large easily dispersed

flakes. However, it seemed that resistance rose to a peak in the lag phase and fell sharply when this ended, only to rise again as the logarithmic phase progressed.

The outstanding feature of the 45° results is that the DRT values were above those for cultures of the same age incubated at 27 and 37°, except for the very youngest cultures. The final resistance in the stationary phase was

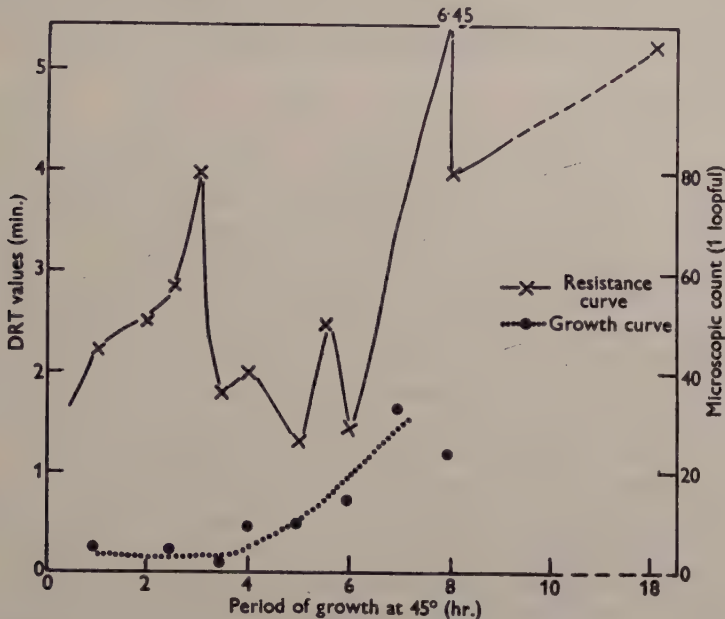


Fig. 7. The growth curve and resistance curve of young cultures of *Strep. faecalis* strain L5 incubated at 45°.

very great indeed, and was retained at this high level until at least 18 hr. Thus, the mature cells in 45° cultures were more resistant than mature cells from 27 and 37° cultures. This unexpected finding agrees with the results of Ellicker & Frazier (1938). Possibly the tendency to form larger groups at the higher incubation temperature may partly explain the higher resistance.

Other strains. Strains L6 and strain C and G behaved like L5 in their general pattern of resistance, showing minor differences, as was to be expected. It was immaterial whether the resistance was expressed as the 99.9 % mortality time or in terms of the DRT. At 45° both L6 and C and G showed the much enhanced resistance that was so marked a feature of the L5 cultures at this temperature.

DISCUSSION

There is a striking similarity between the results reported above for *Strep. faecalis*, and those of Ellicker & Frazier (1938) with *Bact. coli*, in spite of the great difference between these organisms in type and habit of growth. *Strep.*

faecalis forms aggregates more frequently than *Bact. coli*; this property was expected to confuse the present results to some extent. It may have done so, particularly in the initial period of slow death which may have been an artefact due to the presence of clumps, though Jordan & Jacobs (1944) have clearly established that such a period is shown even by *Bact. coli* when the death-rate is made sufficiently slow for samples to be secured before the acceleration has set in. The existence of variations in death-rate during an experiment may be due, as suggested by Hinshelwood (1951), to variations in the resistance of cells according to the stage they have reached in the division cycle.

The present results differ from those of Ellicker & Frazier (1938) only in that lowered temperature did not lead to lowered resistance at all stages of growth. Very young cultures of *Strep. faecalis* at 27° were more resistant than those at 37°, and those at 37° were slightly more resistant than those at 45°. The marked increase in resistance which occurred very soon after transference to fresh medium was striking, as was the sharp fall at the onset of logarithmic growth. In view of these great variations within a short period, and the subsequent changes, it is suggested that the apparently conflicting results of earlier workers, many of whom found decreased resistance in young cultures while a few observed enhanced resistance, may be attributed to differences in technique. Evidently the term 'young culture' needs rigid definition. It is also likely that in different media the time relationships may alter even with one organism, while different organisms would undoubtedly take different times to reach the several phases. Although Ellicker & Frazier's results have been matched so closely on the present work, those workers' curves, both for resistance and growth, show that *Bact. coli* grown in milk took longer to accomplish the full cycle than did *Strep. faecalis* grown in broth.

I am particularly grateful to Dr S. E. Jacobs of Imperial College, London, for his immense help in the preparation of this paper. I would also like to thank Dr T. Richards of the University of Reading for his interest and advice throughout the work and Mrs Atkins, the Librarian of King's College of Household and Social Science, for her help in obtaining literature.

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(Received 4 March 1952)

The Effect of Carbon Dioxide Pressure on a Bacterial Decarboxylase System

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SUMMARY: The effect of CO_2 on a decarboxylase system of *Streptococcus diacetilactis* has been investigated. It was found that the amount of CO_2 produced by a culture of the living organism depended upon the proportion of CO_2 present in the atmosphere. Under minimum p_{CO_2} the production of CO_2 by the organism was stimulated while accumulation of CO_2 repressed the formation of the gas. It also appeared that the presence of excess of other products of reaction repressed the production of CO_2 . An explanation of these phenomena in terms of the law of mass action is advanced.

The effect of CO_2 upon the production of decarboxylase by the organism was also investigated. There was some indication that the composition of the gaseous environment influenced the production of decarboxylase.

During an investigation of the heat stability of an enzyme system of *Streptococcus diacetilactis* (Oliver, 1952) it was noticed that the amount of carbon dioxide produced by a culture of the organism varied according to whether the CO_2 was continuously removed or was allowed to accumulate under pressure in a closed culture apparatus. Where an enzymic reaction is readily reversible it is to be expected that the rate of reaction and the final end point will be influenced by accumulation of end products which, according to the law of mass action, will tend to drive the process in the reverse direction. Where one of the end products is a gas (as in the case under consideration) it may be allowed to escape into the atmosphere, as in a cotton-wool plugged flask; it may be removed from a closed system by absorption in alkali; or it may be confined in a sealed culture vessel with the production of pressures higher than atmospheric. The full implications of these variations in conditions of culture do not seem to have been appreciated by many workers who have investigated the production of CO_2 by plant and animal tissue and by micro-organisms, since manometric, gasometric, gas-entrainment and gas-absorption techniques have been used without any allowance for their possibly different effects on the course of the reactions studied.

In the present paper evidence is presented tending to show that with *Strep. diacetilactis* the equilibrium point of the enzymic reaction by which CO_2 is produced is influenced according to the law of mass action by CO_2 pressure, and further that the amount of enzyme formed by the organism may vary according to the gaseous environment of the growing culture.

MATERIALS AND METHODS

A culture of *Strep. diacetilactis*, strain DL, as described in a previous paper (Oliver, 1952) was used as the test organism, the culture media also being as described in that paper. The organism grew readily in sterilized skim milk

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at 22°, clotting the milk within 24 hr. with the formation of *c.* 0.8% titratable acid (calculated as lactic acid). It should be emphasized that in all the experiments described, active multiplication of the *Strep. diacetylactis* ceased in 24–36 hr. and that the CO₂ production observed for the greater part of the experimental period was a result of the metabolic action of organisms maintained in a non-proliferating condition by the high acid concentration in the culture medium.

Carbon dioxide production was estimated as described in a previous paper (Oliver, 1952). Where it was desired to observe the effect of accumulation of CO₂ over the culture a short length of pressure tubing was fitted between the culture and baryta flasks so that the culture could be sealed off from the absorbent by a screw clip. When the clip was released the gases were bubbled through baryta solution and the CO₂ estimated in the usual manner. In this apparatus the volume of air available to the sealed-off culture was only half that available in the original apparatus where culture flask and absorption flask were open to one another from the start of the experiment. It was found, however, that p_{CO_2} is not critical for this particular enzyme system, for on excluding air by placing a layer of paraffin oil over the culture the total volume of CO₂ produced did not decrease, although the end point was reached a little later than under fully aerobic conditions.

RESULTS

Effect of CO₂ pressure on CO₂ production at 22°

Growth of *Strep. diacetylactis* at 22° in milk and in citrate broth in an apparatus in which CO₂ was continuously absorbed by 0.1N-baryta gave volumes of CO₂ as recorded in Table 1. These figures represent the average result obtained in many experiments, there being only small variations from one culture to another. It will be noted that the total amount of CO₂ increased up to 10 days

Table 1. Carbon dioxide produced by 100 ml. culture of *Strep. diacetylactis* at 22° in an atmosphere in contact with baryta initially 0.1N (average figures)

Medium	Days							
	1	2	3	4	5	6	7	10
	CO ₂ production (ml. N.T.P.)							
Milk	11	21	29	34	38	40	43	46
Broth + 0.01M-citrate	13	30	41	48	52	54	55	56

(after which no appreciable increase occurred) and that gas production was higher in a citrate broth medium than in milk. The p_{CO_2} under these control experimental conditions (over 0.1N alkali) is not accurately known, but it is very low since Dee (1945) derived a figure of 10^{-11} atmospheres for p_{CO_2} over a solution containing *c.* 0.25N-NaOH + 0.25N-Na₂CO₃.

When cultures of *Strep. diacetylactis* were grown in milk or in broth in a type of apparatus in which the culture flask was sealed off from the absorption

flask the volume of CO_2 produced was greatly reduced. Table 2 gives the results of a typical experiment in which culture flasks of two different sizes (containing the same volumes of culture) were used. It is clear that the sealing of the culture flasks decreased the CO_2 production as compared with the control, and that the smaller the gas space above the culture the greater the decrease in CO_2 production. As mentioned above, the volume of O_2

Table 2. *Carbon dioxide produced in 4 days at 22° by Strep. diacetylactis in 100 ml. of milk or citrate broth in sealed vessels of different sizes*

Medium	Gas space in sealed vessel		Unsealed control
	c. 40 ml.	c. 400 ml.	
	CO ₂ produced (ml. N.T.P.)		
Milk	2.0	7.9	85
Broth + citrate 0.01 M	5.7	19.8	47.5

available to the culture is not significant so far as the action of the CO_2 -producing enzyme is concerned. Furthermore, the density of the bacterial population and the total titratable acidity were found to be the same for all the cultures in a given medium irrespective of p_{O_2} or p_{CO_2} . This shows that the phenomenon is not due to a change in the rate of multiplication or in the main metabolic activity of the organism. The most reasonable hypothesis is that the equilibrium point of the enzymic reaction concerned in the formation of CO_2 is altered by CO_2 pressure according to the law of mass action.

Alteration of the equilibrium position by other end products

In a reaction of the type $\text{A} \rightleftharpoons \text{B} + \text{CO}_2$ the removal or addition of either B or CO_2 should affect the equilibrium in a similar way. In practice, it is easier to alter the concentration of the product in the gas phase (i.e. CO_2) than a product B in the liquid phase. Moreover, the exact course of the reaction by which diacetyl and CO_2 are produced by *Strep. diacetylactis* is not known, and hence the identity of B is unknown. However, it is reasonable to assume that the cell-free filtrate from the cultures will contain product B. On this assumption the effect on CO_2 production of adding Seitz-filtered whey, from a 8-day culture of *Strep. diacetylactis* grown over alkali, to a pasteurized culture of the same age and grown under the same conditions was examined. It had previously been shown (Oliver, 1952) that the decarboxylase system of the organism was stable beyond the temperature of pasteurization and would continue to function unabated after the organism had been killed. Thus the use of a culture killed by heating at 80° for 5 min. excluded the possibility that any observed decrease in CO_2 formation might be due to inhibition of the normal metabolic processes of the resting organism by the added products of its metabolism. Table 3 gives the results of a typical experiment in which a pasteurized milk culture of *Strep. diacetylactis* was incubated at 22° in a CO_2 -absorbing apparatus with and without the addition of Seitz-filtered

whey. It is clear that the presence of sterile whey in the pasteurized culture resulted in a decrease in CO₂ production to an extent depending on the proportion of whey added. The results are consistent with the hypothesis that metabolic products other than CO₂ are able to alter the equilibrium point of the reaction in which CO₂ is one of the end products.

Table 3. Carbon dioxide produced by pasteurized 3-day milk cultures of *Strep. diacetylactis* with and without additions of Seitz filtered whey from a similar culture

	CO ₂ produced in 4 days at 22° (ml. N.T.P.)
3-day culture heated at 80° for 5 min.	18.0
3-day culture + 5 ml. whey	11.1
3-day culture + 10 ml. whey	5.4
10 ml. whey in sterilized milk	2.0

Effect of CO₂ on the amount of decarboxylase produced by bacteria

Exposure of a growing culture of *Strep. diacetylactis* to carbon dioxide was found to influence the CO₂-producing capacity of the culture during a subsequent incubation period under conditions in which any CO₂ formed was immediately absorbed in baryta. Cultures in milk and in citrate broth were grown at 22° in three different sets of conditions; in flasks sealed with a screw clip, in flasks plugged with cotton wool, and in flasks attached to an absorption flask containing baryta. These cultures were thus under high, medium and minimum CO₂ pressure respectively during the preliminary growth period. After varying times under these conditions all the cultures were connected to absorption flasks containing alkali, and the incubation at 22° was continued until 7 days had elapsed from the start of the experiment. Table 4 gives a typical series of results. It will be observed that as compared

Table 4. Carbon dioxide produced by *Strep. diacetylactis* at 22° after preliminary growth for various periods under various CO₂ tensions

		Preliminary growth period (hr.)				
		18	24	48	72	96
		CO ₂ produced in subsequent 4 days at 22° (ml. N.T.P.)				
Conditions of preliminary growth	Medium					
Sealed flasks	Milk	28.1	10.9	3.0	1.7	0.6
	Broth	38.4	14.3	3.2	1.8	Nil
Cotton wool plugged flasks	Milk	31.3	22.5	15.0	9.7	5.0
	Broth	44.2	32.3	14.0	5.0	2.4
Control connected to CO ₂ absorption flasks from beginning	Milk	34.3	32.6	21.8	13.0	8.0
	Broth	46.7	42.7	24.2	14.0	7.5

with the control values (those obtained from the cultures grown throughout with continuous absorption of CO₂) the values for cultures grown under medium and high p_{CO_2} during a preliminary period were always significantly

lower, indicating that pressure of CO_2 on a culture tends to decrease its capacity for CO_2 production even when the pressure is subsequently removed.

These results suggest that the effect of CO_2 on *Strep. diacetylactis* is to inhibit synthesis of the decarboxylase. Under optimal conditions a smaller amount of catalyst should finally give the same total CO_2 production as a larger amount, although in a longer time, but in bacterial cultures in which various metabolic products have accumulated the enzymes suffer gradual destruction during the course of their action and hence the volume of CO_2 produced should correspond in some degree with the amount of enzyme formed by the organism.

The concept of evocation of specific enzyme activities in micro-organisms in response to variations in environment is accepted by many workers. Hauka & Koessler (1924) suggested that micro-organisms produce amino-acid decarboxylases in response to an over-acid environment 'as a protective measure when accumulation of hydrogen ions within the organism's protoplasm is incompatible with its normal life process'. Gale (1946) suggested that, since CO_2 is sometimes an essential metabolite, decarboxylases may be formed and produce CO_2 in growth conditions where retention of CO_2 in a medium is limited by solubility considerations. A strongly acid culture, such as is produced by *Strep. diacetylactis* in 24–36 hr., from which CO_2 is being continuously withdrawn fulfils these conditions for increased production of decarboxylase. The increased amount of enzyme formed during the earlier period would then be expected to result in a greater CO_2 production during the resting period. Conversely, a culture in which p_{CO_2} is allowed to increase during the early growth period may be expected to decrease its production of decarboxylase, this being reflected subsequently in a diminished CO_2 output.

It would obviously be desirable to investigate the problem further, using cell-free extracts from bacterial material obtained by centrifuging cultures grown under various CO_2 tensions and to compare decarboxylase activity under conditions of known substrate concentration. This has not been possible so far, in the absence of a satisfactory technique for preparing an active extract. Several methods of grinding centrifuged bacteria were tried, but it was found that the extracts obtained lost their decarboxylase activity within a few minutes. Evidently the decarboxylase was very unstable apart from the whole bacteria cell, or in a medium differing greatly from that in which it was produced.

DISCUSSION

Although studies have been made of the effect of accumulation of CO_2 in a closed system on the respiration of plant tissues and on the metabolic activities of micro-organisms little attention has been paid to the techniques used for estimation of CO_2 in such experiments. Willaman & Beaumont (1928), working with plant tissues, found that the accumulation of CO_2 resulted in a logarithmic decrease in CO_2 output. Hassouna & Allen (1939) investigated the gas production of lactic acid bacteria using a gasometric and an absorption technique. In some cases they observed no evolution of gas with the gaso-

metric method while the absorption technique recorded a significant CO₂ production. They attributed this anomaly to the solubility of CO₂ in the medium but it may well have been due to suppression of CO₂ formation in the gasometric apparatus. Osterwalder & Jenny (1939) found that the fermentation of wine was stopped by the accumulation of CO₂ in a closed vessel but, because they found that an increase in total pressure had a similar though smaller effect, they did not consider p_{CO_2} as having a specific effect on the fermentation. Increased atmospheric pressure results in a higher p_{CO_2} at the site of reaction, so an explanation in terms of the law of mass action is valid. Kidd & West (1945) reported that 10 % of CO₂ in the atmosphere suppressed CO₂ production in apples by 45 %. Zhuravskii (1939) observed suppression of citric acid formation and of mycelial growth by an atmosphere containing 20 % of CO₂. A pathway commonly postulated for citric acid formation (Sumner & Somers, 1947) involves condensation of oxaloacetate and pyruvate to form an intermediate which is oxidized and decarboxylated to citrate, so it might be expected that a high p_{CO_2} would suppress the last (decarboxylative) stage of the reaction.

Foster & Davis (1949) found 'that high CO₂ concentrations specifically inhibit fumarate formation under the above conditions (CO₂ fixation via the Wood-Werkman reaction) and that oxaloacetate decarboxylase activity of the mold is also inhibited by high CO₂ tensions, suggesting that fumarate formation is blocked at the pyruvate + CO₂ stage'. Suppression by CO₂ of decarboxylation by isolated oxaloacetate decarboxylase is to be expected on the basis of the mass law so that the term 'specific inhibition' of the decarboxylation is unnecessary. The scheme of reactions proposed by Foster & Davis for anaerobic consumption of glucose by *Rhizopus nigricans* involves a preliminary conversion to pyruvate, which may then be reduced in one of three ways: without decarboxylation, yielding lactate; after decarboxylation, yielding ethanol; and after carboxylation, yielding fumarate via malate. A high p_{CO_2} was also found to inhibit the production of ethanol, but not that of lactate, which is normally produced in small amount only. The substantial inhibition of ethanol formation and of glucose consumption in the presence of CO₂ may indicate that the whole series of reactions is being forced back via the route $\text{CH}_3\text{CHO} + \text{CO}_2 \rightleftharpoons \text{pyruvate} \rightleftharpoons \text{hexose}$. A knowledge of the energy requirements of the various reactions should be helpful in understanding why the pyruvate which is formed is converted to ethanol rather than to oxaloacetate. The suppression of alcoholic fermentation by CO₂ is often accompanied by a decrease in the rate of growth of the organism, this being in contrast with the effect on *Strep. diacetylactis* where growth is not retarded. In the former case ethanol and CO₂ are the end products of the main metabolic pathway and energy source, while in the latter the production of diacetyl and CO₂ results from a relatively unimportant side reaction and the main energy source (the lactic acid fermentation) does not involve CO₂ production.

It thus appears that, depending upon the reactions involved, CO₂ pressure in a closed system could influence the production of CO₂ and the growth of a micro-organism in the following ways: (i) suppression of CO₂ production

and of growth of the organism through a mass action effect where production of CO_2 is involved in the main reactions brought about by the organism in the medium; (ii) suppression of CO_2 production without suppression of growth through a mass action effect on a reaction which is not essential to the growth of the organism; (iii) suppression of growth of the organism due to a non-specific effect of CO_2 acting as an acid with recovery to normal on removal of pressure. Whichever applies in a given case it is obviously important that the method of estimating CO_2 shall be specified and that there shall be an appreciation of the possible effect of CO_2 pressure on the results obtained. In a large majority of cases it appears that the simplest hypothesis to explain the effect of CO_2 in a closed system is the law of mass action. Since synthesis of adaptive enzymes can be stimulated by the presence in the medium of appropriate substrates, it might be expected that enzyme synthesis would be suppressed by presence of excess of the products of reactions. This would apply equally to gases and to substances in solution. No observations on this point seem to have been made hitherto, but the evidence presented here, although not unequivocal, does lend support to this hypothesis.

The author gratefully acknowledges Dr H. R. Whitehead's helpful criticism and advice throughout the investigation.

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(Received 7 April 1952)

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The Effects of Chemicals on the Recombination Rate in *Bacterium coli*

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SUMMARY: The rate of prototroph formation as a result of gene recombination in multiple mutants of *Bacterium coli*, strain K-12, is altered by addition of various chemicals to the nutrient substrate in which the recombining strains are grown. There is some correlation between the appearance of filamentous forms of the organism and the rate of gene recombination.

The recombination of genetic factors in *Bacterium coli*, strain K-12, and their segregation into prototroph recombinants as reported by Lederberg (1947), has been confirmed by several workers. Nelson (1951) has applied statistical methods to show this phenomenon to be a true recombination. Haas, Wyss & Stone (1948) found that ultraviolet irradiation of the recombining strains produced a marked increase in the number of prototrophs formed, and they reported a parallel increase in the number of filamentous 'large forms' of cells in the cultures. A similar increase in the rate of formation of prototrophs was reported by Clark, Haas, Stone & Wyss (1950) when the recombining strains were cultured together in substrates that had been treated with hydrogen peroxide or irradiated with ultraviolet light. When the strains were grown together in the presence of sublethal concentrations of sodium azide, an increase was also found. These cultural conditions also caused an increase in the number of filaments, but because of experimental procedures it was impossible to obtain any direct correlation between the rate of recombination and the incidence of appearance of the abnormal forms of *Bact. coli*.

In view of the possible significance of the *Bact. coli* filaments in genetic recombination, it was decided to test the effects of various chemical agents on the recombination rate. These chemicals were chosen on the basis of previous observations which had revealed that various degrees of pleomorphism resulted when they were incorporated into media on which *Bact. coli* was grown.

EXPERIMENTAL

The recombining mutants of *Bact. coli* used were strains Y-53 and 58-161 (kindly furnished by Dr J. Lederberg). Strain Y-53 requires threonine, leucine, and thiamine for growth, and strain 58-161 requires biotin and methionine. Nutrient agar was utilized as a complete medium for determining total viable cell counts, and asparagine glucose mineral salt agar (Lederberg, 1947) was used as a minimal medium for determining prototroph formation.

The soluble chemicals used were made in concentrated solutions and sterilized by filtration through a UF fritted glass filter. Appropriate amounts

of the sterile chemical were aseptically added to 50 ml. samples of nutrient broth to give the desired final concentrations. Compounds which were only slightly soluble in water were added directly to the nutrient broth in the desired concentration and autoclaved with the substrate.

The treated nutrient broth samples were inoculated with 0.1 ml. of a log phase culture of each of the recombining strains. After 24 hr. incubation, the resulting cultures were washed twice by centrifugation, resuspended in 50 ml. of saline, and plated in appropriate dilutions in nutrient agar for total count and in the minimal agar for prototroph count.

The treated media were tested for the presence of possible factors of selection by comparing the rates of growth of each of the deficient strains and the prototrophs in nutrient broth and in nutrient broth in the presence of the chemical. In no case was evidence found that any change in the rate of recombination was caused by this factor of selection.

Each experiment was repeated several times, and in every case the same proportional increase or decrease in the rate of prototroph formation was observed. Thus, although the recombination rate in some cases is not vastly different from that of the control, it is believed that all results are significant.

RESULTS

Microscopic examination of all cultures grown in the presence of the chemicals tested revealed varying degrees of pleomorphism, including in many cases the long filamentous cell forms. However, it was impossible to obtain any direct correlation between the rate of prototroph formation and the incidence of filamentous forms because of the time lag incubation necessary to insure prototroph formation. Table 1 shows the effects of small concentrations of norleucine on the recombination rate. The variation in the prototroph formation with different concentrations of the amino-acid follows the same pattern as found for direct ultraviolet irradiation (Haas *et al.* 1948), indirect ultraviolet irradiation, hydrogen peroxide treatment, and sodium azide treatment (Clark *et al.* 1950).

Table 1. *Effect of norleucine on the recombination rate in Bact. coli*

Concentration ($\mu\text{g./ml.}$)	Total count $\times 10^6$	Prototrophs	Prototrophs/ 10^7 cells
0	299	410	18
100	326	180	6
300	277	820	30
500	412	950	22

The presence of sulphanilamide or sulphanilic acid in the substrate during prototroph formation does not cause any apparent increase in prototrophs, as shown in Table 2. However, as the concentration was increased, the incidence of recombination was steadily decreased. This decreased rate was observed with other compounds and may be of equal significance with the increased rates; also it could be due to a suppression of prototrophs by the parent cells (Grigg, 1952).

The antibiotics, penicillin and streptomycin, were also used in sublethal concentrations to determine their effect on the recombination rate. Penicillin was used in concentrations of 20 and 40 units/ml. and streptomycin was used in a concentration of 1 unit/ml. In the concentrations used, cell clumping made it impossible to obtain good duplicate experiments. However, the data obtained indicated a marked decrease in the rate of prototroph formation. Some clumping was noticed in the presence of other chemicals, but this was negligible, and it is not believed that the cell aggregates were involved in gene recombination since no correlation was found between the degree of clumping and the subsequent rate of recombination. Such a possibility cannot be entirely eliminated, however. It has been suggested by Squires & Fuller (1952) that within limits, cell clumps should be regarded as individual cells.

Table 2. *Effect of sulphanilamide and sulphanilic acid on the recombination rate in Bact. coli*

Chemical	Concentration ($\mu\text{g./ml.}$)	Total count $\times 10^6$	Prototrophs	Prototrophs/ 10^7 cell
None (control)	—	420	500	12
Sulphanilamide	100	355	500	14
Sulphanilamide	500	371	300	8
Sulphanilamide	1000	223	110	5
Sulphanilic acid	1000	370	120	3

Table 3. *Effect of various chemicals on the recombination rate in Bact. coli*

Chemical	Concentration (%)	Total count $\times 10^6$	Prototrophs	Prototrophs/ 10^7 cells
None (control)	—	342	360	11
Methylcholanthrene	Sat.	333	435	13
Acenaphthene	Sat.	288	245	9
1, 2, 5, 6-dibenzanthracene	Sat.	323	260	8
Colchicine	0.5	358	90	3
Colchicine	1.0	312	390	12
Ascorbic acid	0.25	219	255	12
Indole-3-acetic acid	0.001	361	210	6
Potassium nitrate	5.0	23	143	64
Magnesium sulphate	5.0	260	540	21

A group of compounds were chosen because of their carcinogenic activity or their effects on nuclear activity in cells. Of these compounds, only colchicine had any appreciable effect and then only in one concentration used as indicated in Table 3. All of these compounds caused pleomorphic cell formation, but in no instance were any filamentous forms observed. The effect of the colchicine was confirmed on repeated experiments.

Other compounds tested were ascorbic acid, which had no effect, and indole-3-acetic acid, which lowered the rate of prototroph formation. High concentrations of potassium nitrate and magnesium sulphate gave a pronounced increase in rate.

The possibility of induced back-mutation in the recombining strains was

eliminated by testing the strains individually for back-mutation in the presence of each of the chemicals used. In no case was any evidence of back-mutation obtained.

DISCUSSION

In view of the lack of knowledge of the mechanisms involved in genetic recombination in *Bact. coli*, it is difficult to evaluate the possible reasons for the alteration of the rate of prototroph formation when cultural conditions are varied. Only generalized concepts can be used as an explanation of such results. The possibility of a life cycle in bacteria has been discussed in detail by Dienes & Weinberger (1951). The correlation between filamentous forms and recombination rate as reported by Haas *et al.* (1948) would fit well into the concept of a life cycle. The conditions under which the rate of prototroph formation is altered also change the incidence of appearance of filamentous forms, although no direct correlation has been obtained. It is possible that the rate of formation of possible sexual forms will vary depending on the conditions of the substrate.

A second possibility is that recombination involves a transfer of diffusible material across the cell membrane of the living cell. This is particularly plausible in view of the effects that high osmotic pressure has on the recombination rate. Such a mechanism could be considered as an *in vivo* transformation, although a transformation as reported in *Bact. coli* by Boivin (1947) and by Wyss (1950) has not been demonstrated.

Hayes (1952) suggested that a bacteriophage might be involved in the transference of genetic properties in prototroph formation in *Bact. coli*. Weigle & Delbrück (1951) have found that the K-12 strain harbours a phage which can be liberated by small doses of ultraviolet light. It is possible that the rate of release of this phage from the host cell could be altered by the chemical composition of the substrate. If the phage were involved in the gene recombination, this could explain the altered rates.

The recent work of Grigg (1952) must also be considered in the interpretation of data on recombination rate. He demonstrated a suppression of the appearance of mutant forms in the presence of large numbers of the parent population. It is possible that the incidence of the appearance of prototrophs may be dependent on the number of auxotrophic parent cells present in the mixed culture. In these experiments the number of viable cells at the time of plating was essentially equal in all cases except in those experiments concerning potassium nitrate. Thus it would be expected that any effects of the parent cells on the recombinants would also be essentially equal. However, it is impossible to predict what differential changes might occur as a result of the population pressures exerted over the 24 hr. incubation period. These possible complex factors of selection are now being investigated.

The author wishes to acknowledge the helpful suggestions and criticisms of this work by Dr Orville Wyss and Dr Wilson S. Stone at the University of Texas.

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(Received 21 May 1952)

Do Bacteria have Mitotic Spindles, Fusion Tubes and Mitochondria?

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SUMMARY: Cytological studies upon species of *Bacillus*, *Bacterium* and *Mycobacterium* have shown that recent claims to demonstrate true mitotic spindles, mitochondria and fusion tubes in these genera are based upon a misinterpretation of the basophilic growing points and septa commonly found in bacteria, and a misunderstanding of the multicellular structure of *Bacillus* and *Mycobacterium*.

I have often drawn attention to the errors of interpretation which may arise from failure to recognize that many bacteria are divided into numerous, small cells by cross-walls and septa (Bisset, 1948 *a, b*, 1950, 1951 *a*, 1952 *a, b*). This is especially true of *Bacillus* and *Mycobacterium*. These observations are not new, and were clearly illustrated by Knaysi (1929, 1930), who also showed that these septa may appear as small granules on the periphery of the cell. In the present paper it will be shown that, in *B. megaterium*, these granules have been misinterpreted as the centrioles of mitotic spindles. Multicellularity in *Bacillus* was illustrated with great clarity by Robinow (1945), who stated: 'Transverse cell walls are very conspicuous in *B. megaterium*. . .'. The cross-walls were also very clearly demonstrated by Murray & Robinow (1952) in partially disrupted preparations of *B. cereus*. Recently, Cassel (1951) succeeded in demonstrating simultaneously the cell walls and nuclei in *B. megaterium* and in other species of *Bacillus*, showing once more that these bacilli contain four short cells, each with a pair of nuclear bodies typical of vegetative eubacteria (Robinow, 1945; Bisset, 1950).

Despite this evidence of the cytological structure of *B. megaterium*, this organism has recently been described by DeLamater & Mudd (1951), as 'a multinucleate cell', the nuclei of which undergo 'a true, mitotic process'. This interpretation is based upon the appearance of so-called 'centrioles' which, in my opinion, are artefacts derived from the cross-walls and septa. This material was also published simultaneously, with the same evidence, by DeLamater & Hunter (1951) and DeLamater (1951 *a, b*). In the last quoted paper it is also claimed that *B. megaterium* conjugates by means of 'fusion tubes'; this appears to be an interpretation of the bacilli of small diameter which are frequently found among chains of larger organisms. This paper, and a subsequent one (DeLamater, 1952) describe what are claimed to be mitotic figures in micrococci, and a further study of *B. megaterium* (DeLamater & Hunter, 1952) claims to show similar appearances in germinating spores. Here again it is my contention that it is developing septa, in dividing cells, which are responsible for the appearances upon which DeLamater bases his interpretation.

A septate structure was also postulated in *Mycobacterium* by Brieger & Robinow (1947), and demonstrated by Bisset (1949, 1950) and Bisset & Moore (1949). However, Mudd, Winterscheid, DeLamater & Henderson (1951), apparently unaware of these studies, produced literary and micro-chemical observations which led them to suggest that the 'granules' of *Mycobacterium* are really mitochondria. These supposed mitochondria are, in my opinion, complete protoplasts (Bisset, 1949). In a subsequent study Mudd, Brodie, Winterscheid, Hartman, Beutner & McLean (1951) claimed that the stainable areas at the growing tips and points of division of *Bacterium coli* are also mitochondria, and presented micrographs of *B. megaterium* showing a row of large granules, corresponding to the basophilic elements of developing septa, which were again stated to be mitochondria.

The purpose of the present paper is to describe the cytological structures observed in *B. megaterium*, *Bact. coli* and certain *Mycobacterium* species, and to show that the descriptions of mitotic spindles, fusion tubes and mitochondria are based upon misinterpretations of the cytological structure of these bacteria, and on failure to recognize multicellularity where it occurs.

MATERIALS AND METHODS

Four strains of *B. megaterium* and one of a *Mycobacterium* sp. were freshly isolated from soil and dust. One strain each of *M. tuberculosis*, *M. lacticola* and *B. megaterium* were from the National Collection of Type Cultures. One strain of *Bact. coli* was freshly isolated from faeces.

Cultures were grown aerobically at 37°, *B. megaterium* and *Bact. coli* on nutrient agar, the mycobacteria on Löwenstein's egg medium. Preparations of *B. megaterium* were made after 4 hr. incubation, and before the beginning of sporulation. These were stained by the acid Giemsa and tannic acid violet techniques (Robinow, 1945; Bisset, 1950), by thionine instead of Giemsa, as recommended by DeLamater & Mudd (1951), or by thionine + thionyl chloride. For the demonstration of basophilic septa, preparations were stained by Heidenhain's iron alum haematoxylin and were mordanted at room temperature for 12 hr.

Preparations were never permitted to dry at any stage, were unfixed, and sealed in water under a coverslip for examination, which was always conducted immediately. None of the photomicrographs in this paper is of a preparation more than 2-3 hr. old, except those which, for comparison, were dehydrated in freezing alcohol according to the method of DeLamater (1951*a*). The considerable differences between fresh water-mounted material and preparations subjected to dehydration in this manner are commented upon later (cf. Pl. 1, figs. 1, 8 and 9). Gold-shadowed electron micrographs were made of some of the material.

Because of its lipid content *Mycobacterium* is difficult to stain with aqueous dyes. The best results were obtained by defatting (Bisset, 1949) before staining, although this method departed from the principle of keeping the material in water at all stages. These preparations, nevertheless, were always water-mounted for examination.

RESULTS

The cytology of Bacillus megaterium

When stained by tannic acid violet to demonstrate the cell walls *B. megaterium* was seen to be composed of a number of short cells, typically four to a bacillus, separated by cross-walls in various stages of formation. These cross-walls showed either as diffuse lines, presumably representing an incomplete formation of cell wall material, or as single, well-defined lines where the process was complete. The centre wall of a bacillus was frequently double, representing an incipient division (Pl. 1, fig. 1).

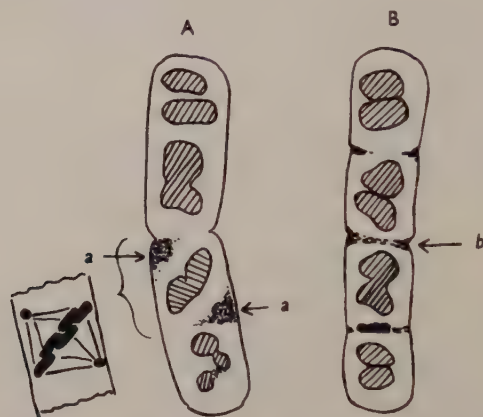


Fig. 1. A, drawing of photomicrograph of *B. megaterium* claimed by DeLamater (1951*b*, and several other papers) to show a 'metaphase spindle' (this interpretation is illustrated, inset, on the left). B, drawing of photomicrograph of *B. megaterium* from Pl. 1, fig. 4, in this paper. The 'centrioles' (a) in DeLamater's figure correspond exactly to the stainable granules at the points of junction of the septa and cell wall (b). Both preparations are stained by acid thionine, but whereas B is mounted in water, A has been subjected to the action of freezing alcohol.

The same organisms, briefly hydrolysed (exact time varying with strain) and then lightly stained by thionine, showed (Pl. 1, fig. 2) that the cell walls did not stain at all; the organism thus appeared very slender, the cross-walls were seen as unstained gaps, with strongly stained bars on each side. These bars are protoplasmic septa which precede and secrete the cell wall material of the cross-walls. In such preparations the nuclear material was not differentiated from the cytoplasm. Similar preparations, more strongly hydrolysed and stained, showed that these basophilic septa stained very deeply at their junctions with the cell wall, thus giving the appearance of discrete granules on the periphery or occasionally at other points on the septum (Pl. 1, fig. 3). These granules are claimed by DeLamater & Mudd (1951) to be the centrioles of mitotic spindles; they are also among the structures claimed by Mudd, Brodie *et al.* (1951) to be mitochondria.

In Pl. 1, figs. 4 and 5, *B. megaterium* is seen fully hydrolysed and stained by acid thionine. The paired nuclear bodies which almost fill the cells, and the basophilic elements of the cross-walls can clearly be seen. In Fig. 1, a drawing

of one of these bacilli is compared with a drawing of a preparation which appears repeatedly (DeLamater, 1951*a, b*; DeLamater & Hunter, 1951; DeLamater & Mudd, 1951) and which is claimed to show a mitotic spindle. The stainable elements of the cross-walls are seen very clearly in Pl. 1, figs. 6 and 7, stained by haematoxylin. In these cases the bacilli appear slender by comparison with Pl. 1, fig. 1, because the mature portions of the cell wall do not stain by these methods.

To study the effect of DeLamater's technique of dehydration in freezing alcohol, by comparison with unfixed fresh material mounted in water, a variety of stained preparations were subjected to this treatment, exactly as described by DeLamater (1951*a*). Results are seen in Pl. 1, figs. 8 and 9, which show the effect of the freezing alcohol technique upon the cell wall preparations of *B. megaterium* stained by tannic acid violet. In some bacilli the multicellular structures shows very clearly, but the organisms are much shrunken in length and distorted. This observation is in accordance with the findings of Møller & Birch-Andersen (1951) on the effect of fixation methods upon bacteria.

The cytology of Mycobacterium

When mycobacteria are stained by acid Giemsa or by a variety of cytochemical methods including the Feulgen technique (see Bisset, 1949, 1950, for literature), a row of granules is seen (Pl. 2, fig. 10), corresponding to the cells into which the bacillus can be observed to be divided when stained by tannic acid violet (Pl. 2, figs. 11 and 12). The basophilic elements in the cross-walls can also be discerned to some extent in Pl. 2, fig. 10. These septa also tend to stain most strongly at points of junction with the cell wall proper (Pl. 2, figs. 11, 12 and 14).

This multicellularity was equally obvious in a filamentous acid-fast organism from the soil (Pl. 2, figs. 14, 15), and in some very short rods from a culture of *M. lacticola* (Pl. 2, fig. 13). Both were subdivided by cross-walls into almost coccid cells. In dried, heat-fixed preparations of *Mycobacterium*, e.g. routine smears stained by Ziehl-Neelsen's stain, the entire contents of these cells, including the putative nuclear bodies, shrink into discrete granules (Bisset, 1949, 1950). These appear to be the 'mitochondria' of Mudd, Winterscheid *et al.* (1951).

The growing points of Bacterium coli

Pl. 2, fig. 16, shows a preparation of *Bact. coli*, unhydrolysed and stained with dilute Giemsa. The stainable areas at the growing tips and at the points of division (Bergersen, 1952; Bisset, 1948*a*, 1951*b*, 1952*b*) are clearly seen. These stain reasonably well with any basic dye, and their appearance is reinforced by the optical illusion of viewing the basophilic cell envelopes across a longer section at these points (Bisset, 1951*a*, 1952*b*), a well-known phenomenon usually referred to as 'bipolar staining'. These organelles appear to be among those claimed as mitochondria by Mudd, Brodie *et al.* (1951) on the ground that they are centres of oxidation-reduction activity. Since they are the areas in which active growth of the cell is occurring, it is perhaps to be

anticipated that they would show marked biochemical activity. Reducing activity at the tips of bacteria was recorded by Bielig, Kausche & Haardick (1949). The identity of these basophilic areas with mitochondria is, in my opinion, most unlikely.

'Fusion tubes' in Bacillus megaterium

Cultures of *B. megaterium* frequently exhibit marked variability of cell form. This was remarked upon by Rettger & Gillespie (1935) who depicted very small cells in series with chains of normal bacilli, but clearly demarcated by cross-walls. Such cells and their cross-walls were often seen in tannic acid violet preparations, especially those of bacilli grown on blood agar. The electron micrographs (Pl. 3, figs. 17, 18) show such appearances. DeLamater (1951*b*) interpreted these structures as fusion tubes. However, as they are not continuous internally, and as most frequently they are attached to larger bacilli only at one end, they appear incapable of performing any such function.

DISCUSSION

The evidence presented upon the cytology of *B. megaterium* and *Mycobacterium* allows little doubt that the basic assumption made by the authors criticized here, namely that these organisms are single, rod-shaped cells, is unjustifiable. They are shown to be subdivided by complex cross-walls, the existence of which invalidates the description by these authors of mitotic spindles, fusion tubes and mitochondria. These misinterpretations have arisen from uncritical reliance upon non-specific staining methods and the use of an unsuitable technique of dehydration.

Analysis of the photographic material presented by DeLamater and his collaborators provides ground for serious criticism. Comparison of the photomicrographs in the various papers published simultaneously by DeLamater, by DeLamater & Mudd and by DeLamater & Hunter shows that all but two of the plates claiming to illustrate metaphase nuclei in *B. megaterium* are taken from the same photomicrograph of the same cell, reproduced at different magnifications, and in one case inverted. An explanation of the resemblance of this cell to a metaphase spindle is given in Fig. 1. Approximately 400 nuclei, claimed to be at other stages of the mitotic cycle, are balanced by this exceedingly flimsy evidence of the most characteristic stage. In the cocci and germinating spores, the 'metaphase' would appear to be an interpretation of two nuclear bodies separated by a developing septum.

Measurement of the organisms illustrated by DeLamater & Mudd (1951), which are printed at a magnification of $\times 4450$, gives a mean length in the reproduction of 27 mm. for twelve bacilli which clearly show the typical number of four nuclear elements, and which are stated to be at 'interphase', 'prophase' and 'metaphase'; thus the mean length occupied by a single nuclear unit is 6.7 mm. The mean length occupied by nuclei from six bacilli, stated to be in 'anaphase' and 'telophase' is also 6.7 mm. Thus there is no correlation between the length of the nuclei and their supposed state of division, or the growth of the cells.

The evidence of Mudd, Winterscheid *et al.* (1951), in support of their interpretations, consists in the demonstration of granules in bacteria, and the claim that their staining reactions prove these to be mitochondria. In the case of *Mycobacterium*, from two to six of these granules are shown in organisms of a configuration which, in the photomicrographs of the present paper, and in the figures of the other workers quoted, have up to six complete cells. It is thus apparent that each of these granules comprises the major portion of the protoplasm of a cell, and may be expected to react with a wide range of reagents, specific for cell components. The basophilic areas at the poles and points of division of *Bact. coli*, described as 'mitochondria' by Mudd, Brodie *et al.* (1951) represent the points of growth of the cells (Bergersen, 1952; Bisset, 1948*a*, 1950, 1951*b*); their biochemical activity is known and is to be expected of such material (Bielig *et al.* 1949). The comparable areas in the multicellular *B. megaterium* are the basophilic elements in the incipient septa and cross-walls. The same structures appear thus to have been described as 'mitochondria' by Mudd in his collaboration with Brodie *et al.* (1951), and as 'centrioles' by DeLamater & Mudd (1951). None of the evidence presented to support the suggestion that any of these structures are mitochondria is adequate to overcome the objection of their obvious functional morphology and complete lack of any resemblance to mitochondria in form and arrangement.

It is thus concluded that these claims to demonstrate mitotic spindles, fusion tubes and mitochondria in bacteria are invalid, and are founded upon inaccurate premises and inadequately controlled methods.

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EXPLANATION OF PLATES

PLATE 1. *Bacillus megatherium*, $\times 8000$

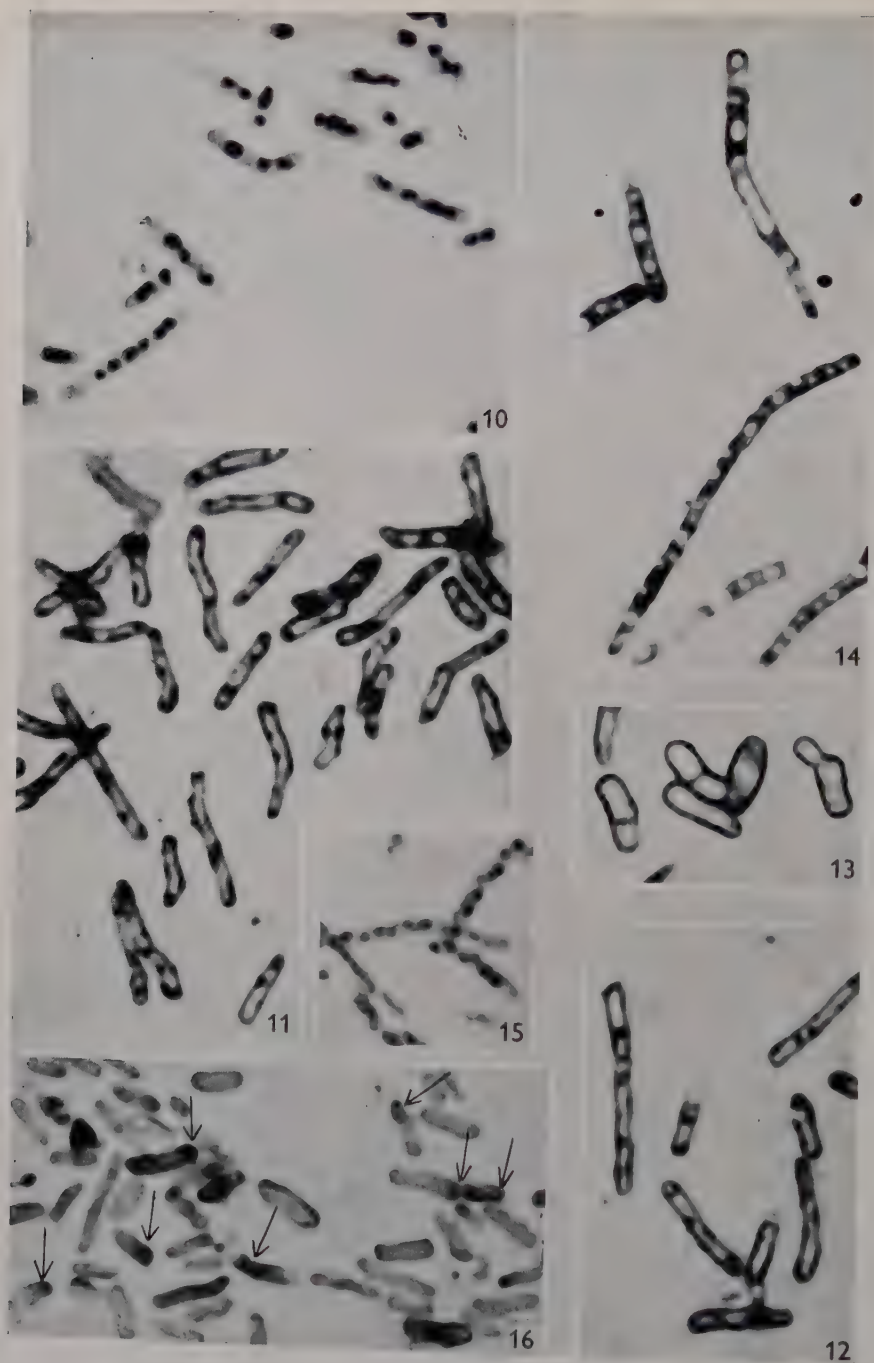
- Fig. 1. Cell walls stained by tannic acid violet. Bacilli typically have four cells, separated by cross-walls.
- Figs. 2, 3. Lightly hydrolysed acid thionine, showing stainable elements in material lining cross-walls. The latter show as unstained gaps. Cell walls are also unstained.
- Figs. 4, 5. Acid thionine, showing cross-walls and paired nuclear bodies.
- Figs. 6, 7. Stainable elements in cross-walls demonstrated by haematoxylin.
- Figs. 8, 9. Tannic acid violet preparations, dehydrated by DeLamater's method, showing shrinkage and distortion, for comparison with water-mounted preparations.

PLATE 2. *Mycobacterium*, $\times 3000$

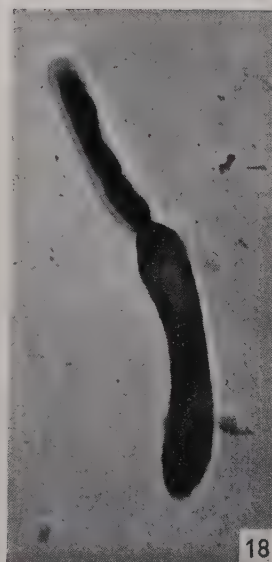
- Fig. 10. *M. lacticola*, acid Giemsa, showing nuclear bodies and some cross-walls.
- Figs. 11, 12. *M. tuberculosis*, cell walls and cross-walls by tannic acid violet. The organism is clearly multicellular.
- Fig. 13. *M. lacticola*, tannic acid violet, showing cross-walls to occur even in very short elements.

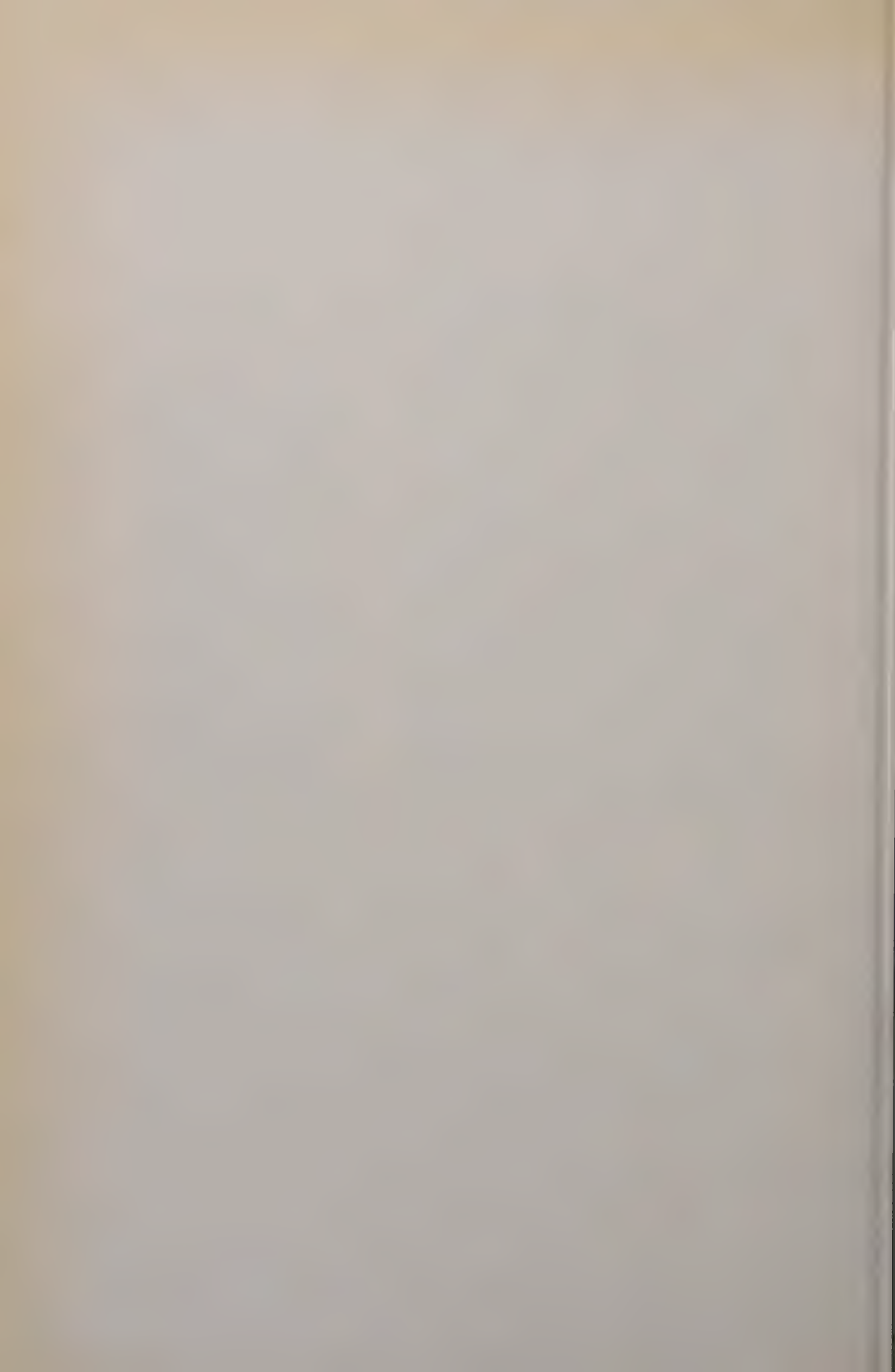


K. A. BISSET—DO BACTERIA HAVE MITOTIC SPINDLES? PLATE 1



K. A. BISSET—DO BACTERIA HAVE MITOTIC SPINDLES? PLATE 2





Figs. 14, 15. Acid-fast organism from soil, tannic acid violet and acid Giemsa respectively. Multicellular, with thick cross-walls.

Fig. 16. *Bacterium coli*, $\times 3000$. Unhydrolysed, stained Giemsa. The stainable surfaces at the growing tips and points of division are seen.

PLATE 3

Figs. 17, 18. Electron micrographs of *B. megaterium*, $\times 7000$, showing small, septate bacilli, in series with those of normal size. Appearances of this type are described by DeLamater as 'fusion tubes'.

(Received 11 June 1952)

Iron and the Nitrifying Bacteria

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SUMMARY: The oxidation of ammonia to nitrite in enrichment cultures of *Nitrosomonas* spp. was hastened by 6 mg. Fe/l. in the medium. Manganese did not replace iron as a stimulant; it was toxic. *N. europaea* (Jensen's strain) and a strain of *Nitrobacter winogradskyi* had, in pure culture, very small absolute requirements for iron; they oxidized ammonia and nitrite respectively in media purified with 8-hydroxyquinoline, and with no iron added. Small amounts of iron hastened the oxidation of ammonia and nitrite; the minimum concentration giving this stimulating effect was 0.1 mg. Fe/l. for the strain of *Nitrosomonas europaea* and 0.3 mg. Fe/l. for the strain of *Nitrobacter winogradskyi*. The optimum amount of iron for oxidation appeared to be about 6 mg./l. for both species. Both species tolerated 112 mg. Fe/l. (c. 0.002M), but oxidation was delayed, markedly in the case of *N. winogradskyi*, by 560 mg./l. (c. 0.01M).

Shortly after Winogradsky first isolated species of bacteria which oxidized, respectively, ammonia to nitrite, and nitrite to nitrate, he reported more rapid oxidation when ferrous sulphate was added to the culture media (Winogradsky & Omeliansky, 1899). Since then iron in some form of combination, and in various amounts, has been added to media used for growing nitrifying bacteria, but the optimum amount of iron and the minimum necessary for growth, have not been determined. It is very difficult to measure the amount of growth of nitrifying bacteria because the cells adhere to solids, including the particles of carbonate in the usual liquid medium; but their activity can be measured by the formation of nitrite from ammonia, or by the disappearance of nitrite as it is oxidized to nitrate. Lees & Meiklejohn (1948) reported that 6 mg. iron/l. increased the rate of nitrite formation in enrichment cultures of *Nitrosomonas* spp.

METHODS

Cultures. Preliminary experiments were done with enrichment cultures, from which a strain of *N. europaea* was subsequently isolated (Meiklejohn, 1950). The ammonia-oxidizing bacterium used in pure culture was a strain of *N. europaea* isolated in Denmark by Dr H. L. Jensen (Jensen, 1950). Pure cultures of a strain of *Nitrobacter winogradskyi*, a nitrite-oxidizing bacterium, were obtained from soil from the Forestry Commission Tree Nursery at Ampthill, Bedfordshire, by two successive platings on nitrite agar. This strain has oval cells, $0.8-1.0 \times 1.0-1.2 \mu$. and is non-motile. It forms zoogloal growth round the chalk particles in liquid medium; cells from a 9-day culture were Gram-negative.

Cultures were grown in the dark at 25°, in 750 ml. flasks containing 100 ml. portions of medium and closed by beakers. The flasks were rinsed with

chromic-sulphuric acid cleaning mixture and with water, and autoclaved with distilled water in them, before use.

Media. Enrichment cultures were grown in a liquid medium containing: $(\text{NH}_4)_2\text{SO}_4$, 0.005 M; NaCl, 0.005 M; KH_2PO_4 , 0.001 M; MgSO_4 , 0.001 M; made up in glass-distilled water, and with 10 g. CaCO_3 /l. added.

For work with pure cultures similar media were used, but were freed as far as possible from heavy metals. Solution A contained: NaCl, 0.6 g.; either $(\text{NH}_4)_2\text{SO}_4$, 1.32 g. (for *Nitrosomonas* sp.) or NaNO_2 , 1.0 g. (for *Nitrobacter* sp.); glass-distilled water, 180 ml.; KH_2PO_4 , 0.1 M solution (previously boiled) 20 ml. The whole solution A was purified with 8-hydroxyquinoline (Waring & Werkman, 1942a). Solution B: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.14 g.; distilled water 50 ml. The complete medium contained: solution A, 100 ml.; solution B, 50 ml.; glass-distilled water, 850 ml.; CaCO_3 , 10 g., and was sterilized by autoclaving at 15 lb. for 15 min.

Iron was added as ferrous sulphate; amounts of 6 mg. Fe/l. and less were autoclaved in the medium, the iron being added as the required volume of a solution containing 0.249 g. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ /100 ml. to the culture flasks. For adding larger amounts of iron a solution containing 2.78 g. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ /100 ml. distilled water was sterilized by filtration through a Ford SB pad, and added to the culture flasks as required after they had been autoclaved.

Manganese (chloride) or copper (sulphate) were added as required to the medium before autoclaving.

Inoculations were made with sterile pipettes; the same volume of liquid culture (0.1–1.0 ml. for different experiments) was added to every flask in the same experiment, in a random order.

Nitrite was estimated colorimetrically with Griess-Ilosvay reagent (Lees & Quastel, 1946).

RESULTS

Enrichment cultures

The effects of various metal supplements on the oxidation of ammonia to nitrite by enrichment cultures containing *Nitrosomonas* sp. are shown in Fig. 1. The addition of 6 mg. Fe/l. (c. 0.0001 M) stimulated the production of nitrite. Manganese did not replace iron as a stimulant, and indeed 1.08 mg. Mn/l. appeared to be poisonous. The effect of manganese was partly counteracted by copper, and almost entirely counteracted by iron; rather more stimulation was given by all three metals together than by iron alone (cf. Lees & Meiklejohn, 1948).

Nitrosomonas europaea

The results of a typical experiment with pure cultures of *N. europaea* (Jensen's strain) are shown in Fig. 2. The accumulation of nitrite, formed by the oxidation of ammonia, was measured in this case. The first point of interest is that nitrite was formed, though slowly, in the control cultures without added iron, although the medium had been purified with 8-hydroxyquinoline

A substantial proportion of the ammonia supplied (about 180 mg.) was converted to nitrite in these control cultures.

Secondly, as in the *Nitrosomonas* enrichment cultures, there was a very marked stimulation of nitrite production by 6 mg. Fe/l. in the pure cultures of this (Jensen's) strain of *N. europaea*.

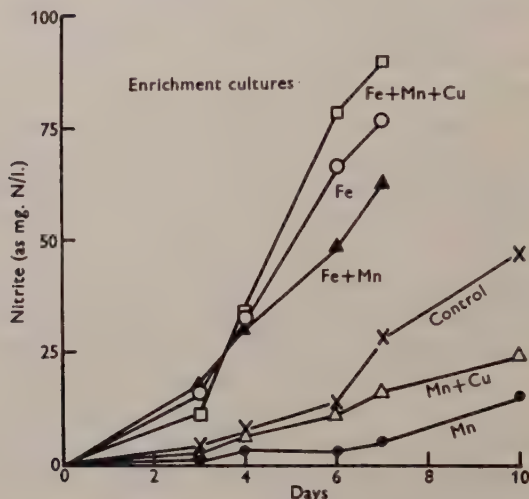


Fig. 1. Nitrite produced from ammonia by enrichment cultures containing *Nitrosomonas* spp. Metals: \times — \times , control (none added); \bigcirc — \bigcirc , iron, 6 mg./l.; \bullet — \bullet , manganese, 1.08 mg./l.; \blacktriangle — \blacktriangle , iron + manganese; \triangle — \triangle , manganese + copper, 0.14 mg./l.; \square — \square , iron, manganese, and copper.

The results of adding smaller amounts of iron (down to 0.05 mg. Fe/l.) are illustrated in Fig. 3. It will be seen that the addition of 0.05 mg. Fe/l. had a negligible effect; definite stimulation was given by 0.1 mg. Fe/l., and the stimulation increased with increasing iron concentration up to 6 mg. Fe/l.

Fig. 3 also emphasizes that the effect of iron operated in the early stages of the oxidation (cf. Fig. 2).

Larger amounts of iron were next used with results shown in Fig. 4; 28, 56 and 112 mg. Fe/l. all stimulated nitrite production, but no more than was obtained with 6 mg. Fe/l. With 560 mg. Fe/l. (c. 0.01M); which gave a heavy brown precipitate in the medium, there was slightly retarded nitrite production as compared with the control.

Nitrobacter winogradskyi

The activity of *N. winogradskyi* cultures was measured by the rate at which nitrite disappeared from the medium. This disappearance was due to oxidation to nitrate; a strongly positive reaction for nitrate was found in every culture at the end of the experiments.

Fig. 5 shows the results of a typical experiment, which are similar to those obtained with the strains of *Nitrosomonas* sp. Oxidation (of nitrite) was completed even in the purified medium with no added iron; 6 mg. Fe/l. accelerated the oxidation of nitrite to nitrate.

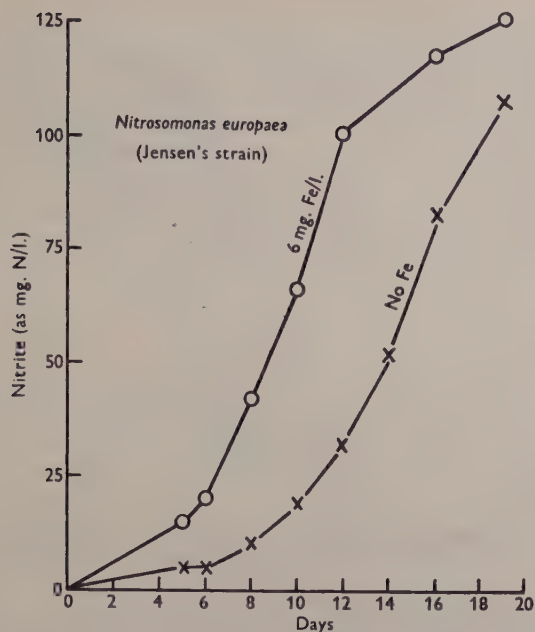


Fig. 2. Nitrite produced from ammonia by a strain of *Nitrosomonas europaea*. x—x, control (no added Fe); o—o, 6 mg. Fe/l. added. (Average values from duplicate cultures.)

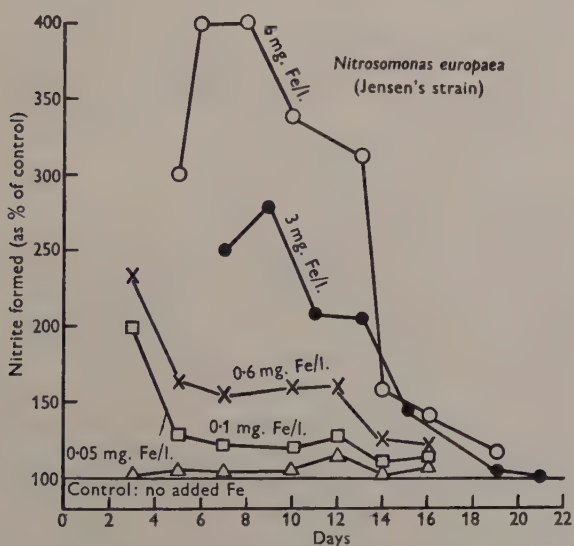


Fig. 3. Effect of different concentrations of Fe on the oxidation of ammonia to nitrite by a strain of *Nitrosomonas europaea*. The nitrite formed is given as percentages of the amount of nitrite in the control at the corresponding time. *N. europaea*, iron added: Δ—Δ, 0.05 mg./l.; □—□, 0.1 mg./l.; x—x, 0.6 mg./l.; ●—●, 3.0 mg./l.; ○—○, 6.0 mg./l. (av. of duplicates).

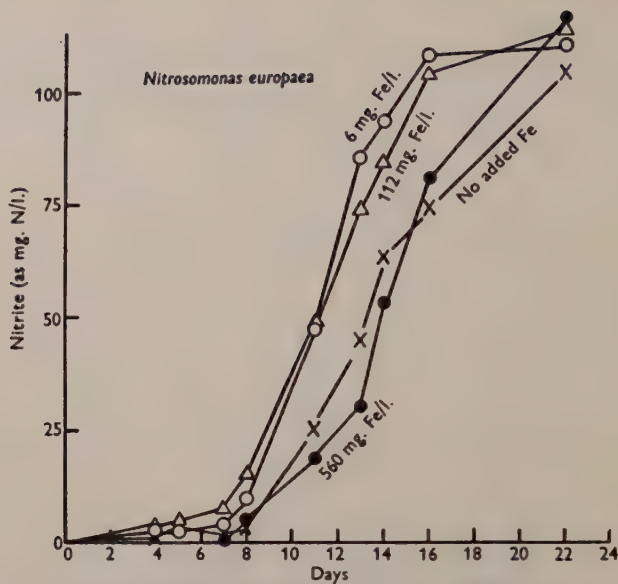


Fig. 4. Effect of large concentration of Fe on oxidation of ammonia to nitrite by *Nitrosomonas europaea*. x—x, control (no added Fe); o—o, 6 mg. Fe/l.; Δ—Δ, 112 mg. Fe/l.; ●—●, 560 mg. Fe/l.

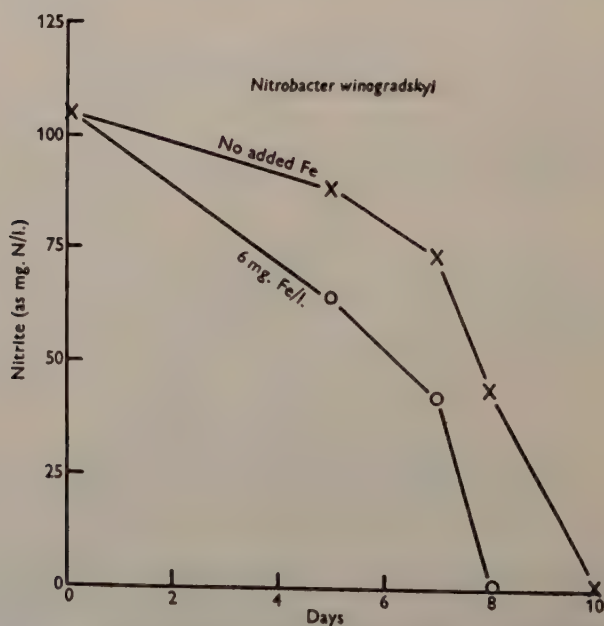


Fig. 5. Oxidation of nitrite to nitrate by a strain of *Nitrobacter winogradskyi*. x—x, control (no added Fe); o—o, 6 mg. Fe/l. (Average values from duplicate cultures.)

The strain of *Nitrobacter winogradskyi* did not respond to such small amounts of iron as did the strains of *Nitrosomonas*. The results of experiments with less than 6 mg. Fe/l. are shown in Fig. 6, where the residual amounts of nitrite (i.e. not oxidized to nitrate) in the treated cultures are shown as percentage of the corresponding figure in the control culture, thus, a value of less than 100 % indicates stimulation of oxidation. No stimulation was produced by 0.1 mg. Fe/l.; stimulation was given by 0.3 mg. Fe/l. and the stimulating effect increased with concentration up to 6 mg. Fe/l.

The strain of *Nitrobacter winogradskyi* used was more sensitive than the *Nitrosomonas* strains to large doses of iron (Fig. 7).

DISCUSSION

The amounts of iron added, by different workers, to media used for growing nitrifying bacteria, have been very various; most early workers used Omeliansky's (1899) medium, which contains c. 80 mg. Fe/l. Jensen (1950) considered this iron concentration too high, and decreased it to 20 mg. Fe/l. in the medium he used for *Nitrosomonas* spp., the same concentration as used by Bömeke (1949). Most American workers, following Gibbs (1919), included 'a trace' of iron in their media. Kingma Boltjes (1935) and Hanks & Weintraub (1936) used 2 mg. Fe/l. for *Nitrosomonas* spp. It is not suggested that all strains of nitrifiers have the same optimum requirements for iron, but it may well be that some of the trouble that has been reported in getting nitrifiers to grow has been due to unsuitable Fe concentrations in the media.

Bömeke (1949) found that not adding iron to his media had no effect on *Nitrobacter* spp.; *Nitrosomonas* sp. strain 44 (later, 1951, classified as *N. oligocarbogenes*) formed, in a month, 82 % of the amount of nitrite formed when iron was supplied; but *Nitrosomonas* sp. strain 32, which he regarded as authentic *N. europaea*, did not form nitrite unless iron was added.

The present work shows that the absolute requirements for iron, of the strains investigated, are very small. Oxidation of ammonia and of nitrite went to completion in media purified with 8-hydroxyquinoline, and without added iron. But small amounts of iron stimulated oxidation by both species; the lowest concentration giving this effect was 0.1 mg. Fe/l. for the strains of *Nitrosomonas* sp. and 0.3 mg. Fe/l. for the strain of *Nitrobacter winogradskyi*. The optimum amount of iron for the oxidations by all the strains appeared to be about 6 mg. Fe/l. which is unusually high for bacteria. Waring & Werkman (1942b) reported that maximum growth of *Aerobacter aerogenes*, *A. indologenes*, *Klebsiella pneumoniae* and *Bacterium coli* was obtained with only 0.02–0.03 mg. Fe/l.; *Pseudomonas aeruginosa* (which has a complete cytochrome system) needed 0.09–0.10 mg. Fe/l. for maximum growth; *Serratia marcescens* needed 0.03 mg. Fe/l. for growth, and 0.3 mg. Fe/l. for maximum pigment production. Young, Begg & Pentz (1944) found that *Bact. coli* needed 0.5–1.4 mg. Fe/l. for optimum growth, when enough magnesium was supplied. McNaught, Owen & Smith (1950) found that the mixed bacteria of the cow's rumen needed 1–2 mg. Fe/l. for good growth.

The optimum value of 6 mg. Fe/l. for the nitrifiers here reported is of the

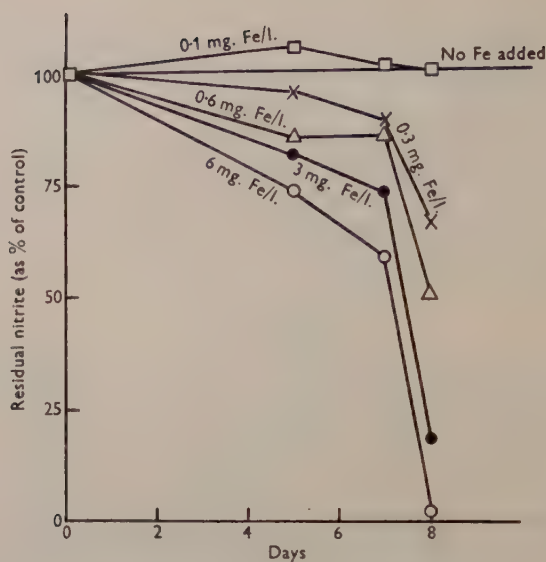


Fig. 6. Oxidation of nitrite to nitrate by a strain of *Nitrobacter winogradskyi*. Residual nitrite shown as percentage of that in the control with no added Fe, at corresponding times. \square — \square , 0.1 mg. Fe/l.; \times — \times , 0.3 mg. Fe/l.; \triangle — \triangle , 0.6 mg. Fe/l.; \bullet — \bullet , 3.0 mg. Fe/l.; \circ — \circ , 6.0 mg. Fe/l. (Average of duplicates.) The nitrite content of the control cultures with no added Fe is shown as the solid horizontal line at 100%.

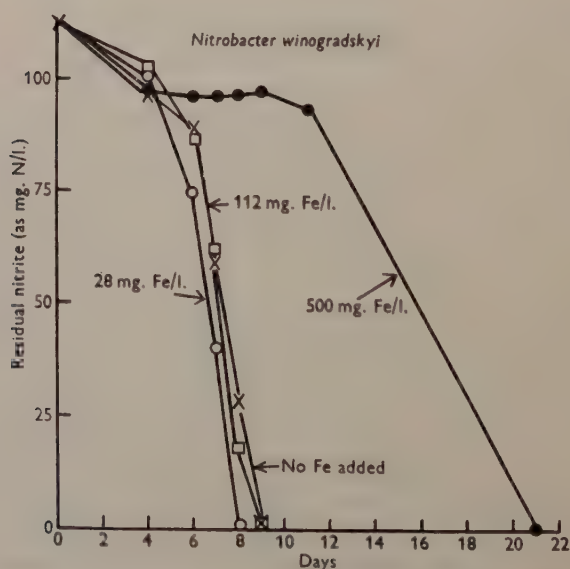


Fig. 7. Oxidation of nitrite to nitrate by a strain of *Nitrobacter winogradskyi*. \times — \times , control (no Fe added); \circ — \circ , 28 mg. Fe/l.; \square — \square , 112 mg. Fe/l.; \bullet — \bullet , 560 mg. Fe/l.

order found for the growth of *Aspergillus* spp. (Steinberg, 1919; Roberg, 1928). The nitrifiers appear also able to tolerate relatively large quantities of iron, up to 112 mg. Fe/l. Waring & Werkman (1942*b*) reported some decrease in growth of the species of bacteria they examined in presence of as little as 3–4 mg. Fe/l. when the iron was supplied as an inorganic salt; iron supplied as citrate was tolerated at 100 mg. Fe/l. Iron at 0.01 M, reported by Meyerhof (1916) to inhibit respiration of the *Nitrosomonas* strains he examined, was found in the present work to delay oxidation by the *Nitrosomonas* strains slightly, and by the strain of *Nitrobacter winogradskyi* more severely.

I wish to thank Dr H. L. Jensen for sending cultures of his strain of *Nitrosomonas europaea*; Dr H. G. Thornton, F.R.S., for his encouragement; Miss E. McCall, Miss F. Zweig and Miss A. Roe for technical help.

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(Received 14 June 1952)

A Simple Method for Producing Microcultures in Hanging Drops with special reference to Organisms Utilizing Oils

By D. M. WEBLEY

WITH A NOTE BY V. C. FARMER

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SUMMARY: An aseptic technique is described for obtaining small hanging drops on the surface of coverslips, using only apparatus that is cheap and easy to obtain. The technique has been used to demonstrate the growth of *Nocardia salmonicolor* on liquid paraffin droplets.

Of all the methods used for studying the growth and development of micro-organisms in liquid culture in undisturbed condition the hanging drop is probably the best. The ordinary hanging-drop method, as used for routine examination of gross bacterial morphology and motility, yields unavoidably large drops and is unsuited to the study of the growth and development of a few cells. Microbiologists requiring more stringent control of drop size have turned to the technique of micromanipulation, but for the average worker the apparatus required is too time-consuming and tedious to construct. The technique described below is offered for those workers who do not require the refinements of micromanipulation methods. The apparatus required is cheap and easy to obtain.

METHOD

The method consists of spraying a suspension of organisms, in the medium in which they are to be studied, on to the surface of coverslips. The suspension is made of such a density that the droplets formed on the coverslip surface contain a few cells each. The coverslip is then inverted over a well slide containing liquid paraffin and incubated at the required temperature.

Description of apparatus and technique

The spray. An all-glass spray of the type depicted in Fig. 1 (manufactured by Parke, Davis and Co., Hounslow, Middlesex, under the trade name Glaseptic Nebuliser) is used. It is plugged as shown and sterilized empty, Fig. 1, A. The suspension of organisms is introduced aseptically at the top of the spray. The squeeze bulb is then attached. Another type of spray manufactured by Parke, Davis and Co. has a slightly different design (Fig. 1, B). If this is used it is an advantage to attach a short piece of glass tubing carrying a cotton-wool plug (see Fig. 1, B) to the side arm by a short piece of rubber tubing before sterilization. In all other essentials the spray is the same as the type depicted in Fig. 1, A.

Moist chamber. Owing to rapid evaporation of microdrops, it is necessary

to spray in an enclosed humid atmosphere. Fig. 2 illustrates a type of moist chamber which has given good results. It is made from: (1) A porcelain lampshade (*A*) with a small piece removed to allow free passage of the rubber tubing attaching the squeeze bulb to the side of the spray; (2) a cylindrical glass jar open at both ends (*B*); (3) a circular glass plate (*C*) with a hole of about $\frac{1}{4}$ in. in diameter drilled in the centre; and (4) a glass ring (*D*) ($\frac{5}{8}$ in. in diameter $\times \frac{1}{4}$ in. in height). The parts are assembled as illustrated in Fig. 2.

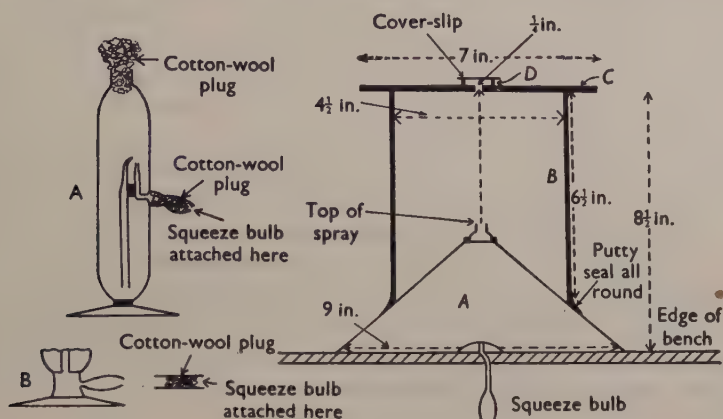


Fig. 1

Fig. 2

B is sealed by a ring of putty to the side of the lampshade and is lined with moist filter-paper. *C* can be sealed with liquid paraffin at its contact with the top edge of *B*; the circular hole in *C* is aligned with the top of the spray when the latter is in position. The glass ring *D* is placed so that the circular hole in *C* is in the centre of the area covered by *D*. *D* is kept in position by liquid paraffin between the surface of *C* and its edge. It is advisable to use a fresh sterile circular ring, *D*, for each experiment. These can be sterilized in Petri dishes. When required, a ring can be sealed in position over the orifice in *C* just before the coverslip is placed in position (see Fig. 2), the surface of *C* being swabbed just prior to the placing of *D* and its coverslip.

Preparation of coverslips. To obtain well-formed droplets with a circular edge it is necessary to coat the coverslips with a water-repellent film. For this purpose, Reyniers (1933) and Gee & Hunt (1928) prepared coverslips with a thin grease film. While this method can be employed quite satisfactorily, it has been found that more consistent results requiring very little experience can be obtained by coating the coverslips with a silicone water-repellent film. For a discussion of the use of silicone water repellents the reader is referred to Gilbert (1951). The method used in this investigation is as follows: no. 1 coverslips $\frac{7}{8}$ in. square are thoroughly cleaned in hot chromic-sulphuric acid cleaning mixture. After removal from the cleaning solution they are well washed by several passages first under running water and finally through distilled water after which they are stored in 50 % ethanol. Before application

of the silicone film, the coverslips are removed from the ethanol and dried with acetone. When completely dry they are treated with a silicone impregnated tissue; 'lens cleaners' (Macleans' Ltd., Brentford, Middlesex) are very satisfactory. The coverslip is placed between the folds of one sheet of the tissue and rubbed with the tissue, using finger and thumb. The same sheet of tissue can be used for several coverslips. The slip is then sterilized by one or two passages through a flame and placed in a sterile Petri dish to cool.

Operation. The spray is placed in position (see Fig. 2), the plug in its neck removed, and the plate *C* and glass ring *D* quickly put in position as previously described. A sterile coverslip is then placed on *D*. When the spray is operated, most of the microdrops which pass through the circular hole in *C* are collected on the coverslip. If the top of the spray is correctly aligned with the orifice in *C* and the glass ring *D*, the droplets will be formed over the central area of the coverslip. The number of droplets formed depends on the operator. When the bulb is working efficiently one or two firm squeezes usually suffice. For ease of operation it is an advantage to allow the squeeze bulb to hang over the edge of the bench, which should be of such a height that one can observe comfortably the formation of the droplets by looking down on the top side of the coverslip while operating the squeeze bulb. When sufficient droplets have formed, the coverslip is quickly placed over the well previously filled with liquid paraffin. This slide is now ready for examination under the microscope.

RESULTS

Pl. 1, fig. 1, gives a visual idea of the droplet size obtained with the technique. As is to be expected, there is a wide variation in size of the microdrops; measurements of droplets formed in this way have ranged from 17 to 170 μ . in diameter.

When using a suspension of organisms it is necessary to adjust the density of the suspension by trial and error so that the required number of cells can be obtained in the desired size drop. If the original suspension has been suitably diluted there is usually little difficulty in finding droplets containing the required number of cells, and it is possible to find droplets containing a single cell, e.g. Pl. 1, fig. 2. The technique is most useful for organisms which give uniform suspensions, and to ensure this it is sometimes necessary to shake the original suspension with sterile glass beads before placing it in the spray. A total volume of 2–5 ml. of suspension is required for the type of spray described.

For organisms which attack liquid paraffin, a small drop of the medium is placed in the bottom of the well. In this case, only the space between the coverslip and edge of the well is sealed with liquid paraffin. In addition, it is an advantage to coat the well with a silicone film to suppress dew-drop formation. Unless the organisms are suspended in distilled water, little diminution in size of the droplets takes place with incubation periods up to 4–5 days (see note). Under these conditions silicone-treated coverslips are much superior to those prepared according to the grease film methods of Reyniers (1933) and Gee & Hunt (1928).

At this stage the technique can only be used for direct observations on the growth and development of cells in undisturbed condition. It is possible to fix and stain the cells using the technique described by de Fonbrune (1948) for his oil chambers. However, owing to the presence of the water-repellent film the cells do not settle on the coverglass in their observed position in the drop, but are pulled to one spot as the aqueous drop evaporates through the paraffin. An exception to this is with cells which have hydrophobic properties. For example, it has been observed with the paraffin-decomposing nocardias, e.g. *Nocardia salmonicolor*, that some cells will migrate to the coverslip at its point of contact with the edge of the drop. Cells in this position will then tend to grow upwards along the air-liquid interface, there being no paraffin in the well. (See also section on growth of *N. salmonicolor* on paraffin droplets.)

In the de Fonbrune method these difficulties do not arise during fixation because the coverglasses used do not need water-repellent films. It is not possible to obtain well-formed droplets on untreated grease-free coverslips with the spray method. The drops formed under these conditions tend to spread out into flat irregular shapes where they hit the coverslip surface. The only way I have been able to fix and stain cells with this method in their original position is to dry in air the untreated coverslips after spraying, and coat them with molten agar media according to the methods of Fleming, Voureka, Kramer & Hughes (1950). With this method, the initial cells of the resulting microculture are more evenly dispersed than when the suspension is, in the ordinary way, spread over the coverslip. After the necessary incubation, fixed and stained preparations can then be made by any suitable method.

Growth of Nocardia salmonicolor on paraffin droplets

N. salmonicolor is known to grow on liquid paraffin as sole carbon source (Erikson, 1949). A suspension of this organism was made by adding to sterile liquid paraffin containing glass beads, a little of the growth of *N. salmonicolor* from the surface of an agar plate (2 % glucose + Czapek salts (nitrate) + 2 % agar). Vigorous shaking by hand was required to obtain a uniform suspension. The paraffin + organism suspension was then introduced into a dry sterile spray. Spraying was carried out in the usual way. The coverslip with its paraffin droplets was then inverted over a sterile well slide, the well being filled beforehand with sterile mineral salt solution (Czapek salts). As the excess mineral salt solution flowed out at the sides of the coverslip it could be taken up with filter paper until the coverslip settled down firmly over the well. Incubation was carried out in moist chambers. When using oils such as liquid paraffin there are very few evaporation troubles; also it is not necessary to apply a water-repellent film to the coverslip beforehand. Pl. 1, fig. 3, shows the growth of the organism after 17–20 hr. incubation at 25° on a paraffin droplet. It was observed that the growth took place at the oil/water interface. A similar experiment was performed in which 1.5 % agar was added to the mineral salt solution; the coverslip was lowered on to the agar medium in the well while the agar was in the molten condition (42°). Pl. 1, fig. 4, gives the result after

17–20 hr. incubation at 25°; the organism has grown away from a droplet between the agar and the under surface of the coverslip; connexion with the oil droplet can be clearly seen. The results depicted in Pl. 1, figs. 3 and 4, demonstrate clearly the mycelial type of initial development for this organism (Erikson, 1949). It is interesting to note that it occurs on liquid paraffin. Similar results have been obtained with vegetable oils, e.g. olive oil, almond oil and castor oil.

DISCUSSION

It must be stressed that the technique presented in this paper is not an alternative for micromanipulation methods. It is obvious that single cell cultures for transplantation cannot be obtained with it, nor is it possible to transfer cells from drop to drop; at present this can only be done by micromanipulation. However, when all that is required is the direct observation of the behaviour and development of a few cells the method should be useful to workers with limited facilities for such purposes at their disposal. In addition, it seems that the technique will be useful for the study of organisms which attack oils.

The author wishes to thank Mrs D. Oxford and Dr G. K. Fraser for their continued interest in this work, and also Miss I. F. Taylor for taking the photographs.

Note

BY V. C. FARMER

The vapour pressure p of a droplet of radius r is greater than that (p_0) of a plane surface, as given by the equation

$$2\gamma M/r\rho RT = \log p/p_0 \approx \Delta p/p_0,$$

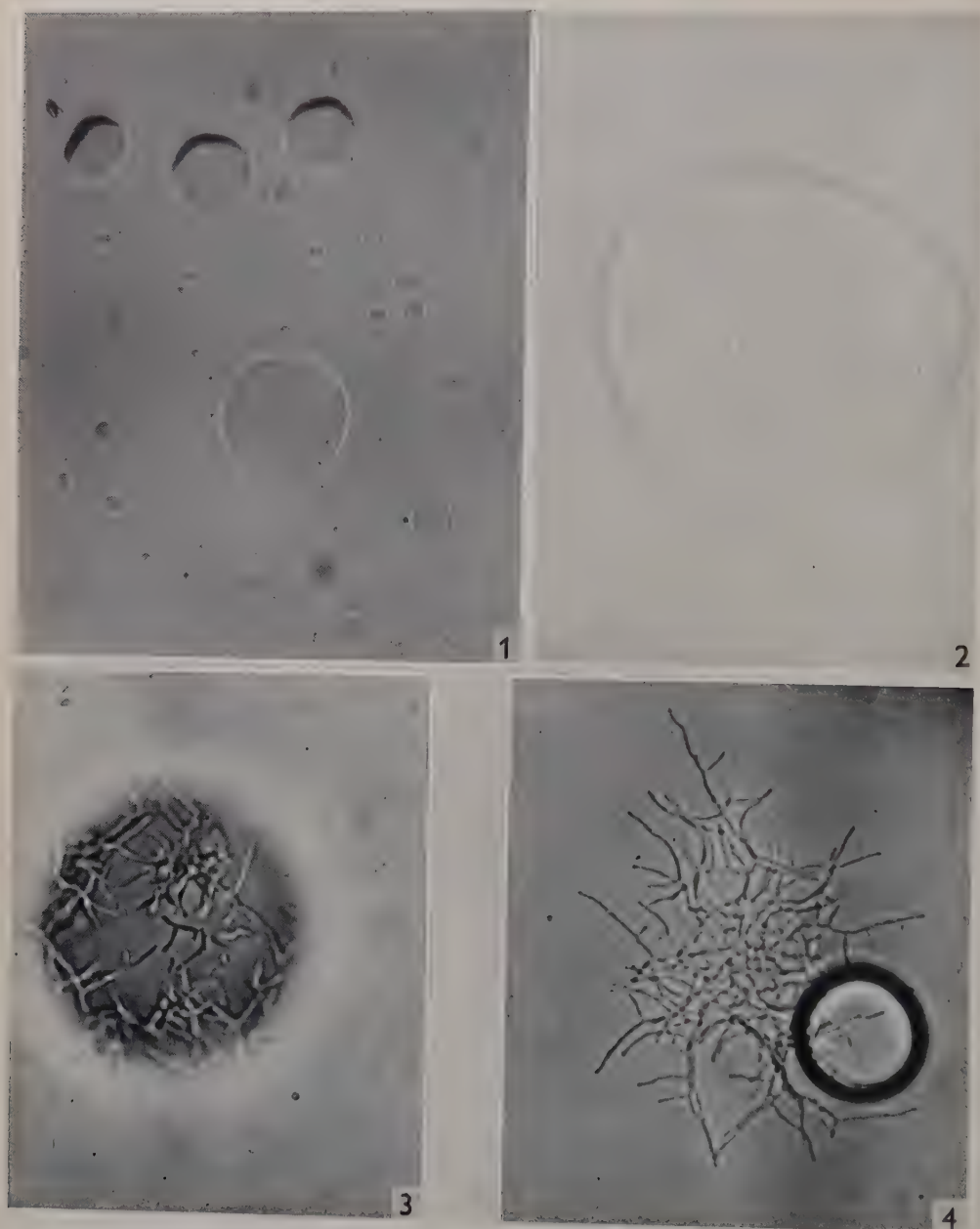
where γ is the surface tension, ρ the density, and M the molecular weight of the liquid, R is the gas constant and T the absolute temperature.

Therefore solvent will be transferred from the droplets on the coverslip to the virtually plane surface of the drop of culture medium in the well of the slide. As the concentration of the solution in the droplet increases, its vapour pressure will fall until equilibrium is reached. Raoult's law gives the change of vapour pressure due to this effect as $\Delta p/p_0 = -\Delta x$, where Δx is the change in mole fraction of the solute.

Thus at equilibrium $\Delta x = 2M/r\rho RT$.

Such changes in temperature (20–40°), density and surface tension (60 to 70 dynes/cm.) as are likely to occur under experimental conditions will not affect Δx by more than 20%. Thus we may calculate that the change in molar fraction will be about 10^{-4} for the smallest droplets ($r = 10\mu$.) or 10^{-5} for the largest ($r = 100\mu$.).

The other important factor is the concentration of the nutrient solution. For instance, the smallest droplets of a 1% glucose solution (molar fraction 10^{-3}) would undergo a 10% change in concentration, and the largest only a 1% change. Any increase in concentration (say by adding inorganic salts) would decrease the effect further.



D. M. WEBLEY—HANGING-DROP MICROCULTURES. PLATE 1

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EXPLANATION OF PLATE

PLATE 1

- Fig. 1. Sterile hanging microdrops (distilled water under liquid paraffin). $\times 80$.
- Fig. 2. Hanging microdrop (distilled water under liquid paraffin) containing a single yeast cell. $\times 680$.
- Fig. 3. *Nocardia salmonicolor* growing on a hanging microdrop of liquid paraffin surrounded with Czapek's salt solution (see text for details).
- Fig. 4. As for fig. 3, but with 1.5 % agar added to the mineral salt solution (for details see text).

(Received 27 June 1952)

Observations on a Transmissible Agent Determining Sexual Differentiation in *Bacterium coli*

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SUMMARY: Analysis of a pair of *Bacterium coli* K-12 mutants which had ceased to show genetic recombination after storage, implicated mutant 58-161, which had previously behaved as a gene donor strain, as the infertile parent. Infertile 58-161 failed to display recombination when crossed with a gene acceptor strain (W-677) but was able to mate successfully with wild-type K-12 and prototrophic recombinant donor strains, i.e. it had become a gene acceptor. The terms 'F+' and 'F-' have been adopted (after Lederberg, Cavalli & Lederberg, 1952) to denote donor and acceptor strains respectively. Growth of either 58-161/F- or W-677/F- in mixed broth culture with 58-161/F+ resulted in the conversion to F+ of up to 75% of re-isolated colonies of the initially F- strain. F- strains converted to F+ by strains of dissimilar genotype showed no phenotypic alteration and, therefore, were not recombinants. Washed, mixed cultures on minimal agar yielded an F+ conversion rate of only 3.6%, while 100% recombinants were F+ under the same conditions. The F+ agent could be transmitted serially through F- strains and was not filterable. While F- \times F- crosses were sterile, F+ \times F- crosses showed maximum fertility. F+ \times F+ crosses were c. 10-20 times less productive than F+ \times F-. The F+ agent had a determining effect on the phenotype of recombinants. Thus, when the F+ F- relationship was reversed in F+ \times F- crosses between the same pair of mutants, almost all recombinants which did not show new patterns of unselected marker characters had the phenotype of the F- parent. Among recombinants from F+ \times F+ crosses, the phenotypes of both parents were represented though not always equally. This effect of F+ on the phenotype of segregants invalidates much of the evidence for genetic linkage in K-12. Reversal of F+ potential in otherwise similar crosses also had a marked effect on the efficiency of prototroph formation on minimal agar supplemented with various growth factors required by one of the parent auxotrophs. A tentative theory of the mechanism of recombination is presented on the basis of this and previous work. This supposes that F+ is a non-lytic infectious agent, harboured by F+ cells and absent from F- cells, which becomes effectively associated with a part (or parts) of the chromosomes of a small proportion of the cells it inhabits. The F+ agent thus acts as a gene carrier in the transfer of genetic material from F+ to F- cells.

Genetic recombination in bacteria was first demonstrated in the K-12 strain of *Bacterium coli* (Lederberg & Tatum, 1946; Tatum & Lederberg, 1947) and its occurrence in this strain has since been confirmed by many workers. Lederberg, Cavalli & Lederberg (1952) have recently reported some forty strains of *Bact. coli* from over 2000 separate isolates which either out-cross with K-12 mutants or show inter-fertility, so that genetic recombination may be considered a not uncommon feature of this species when such potential incompatibility factors as colicine production and lysogenicity are taken into account (Lederberg, 1951).

When irradiation-induced mutants of K-12, having two or more comple-

mentary nutritional dependencies, were grown together in a complete medium and the mixed culture washed free of nutrients and plated in minimal agar, one colony developed for every 10^6 – 10^7 cells seeded (Lederberg & Tatum, 1946). The cells of these colonies were prototrophic, i.e. they resembled the wild-type strain in having acquired the inheritable capacity to synthesize all the growth factors upon which the mutant strains were dependent. The mutant strains did not show mutational reversion to prototrophism when cultured separately in minimal agar. By a further series of mutational steps the auxotrophic 'parent' mutants were labelled with a number of complementary differences in characters, such as fermentative capacity and phage resistance or sensitivity, which were not selected by growth in minimal medium. Analysis revealed that the patterns of these unselected marker characters in prototrophs frequently differed from that in either mutant, while their distribution afforded plausible evidence of linkage (Lederberg, 1947). Since culture filtrates of either mutant were incompetent in recombination (Davis, 1950) it seemed reasonable to assume as a working hypothesis that the genetic rearrangements found in prototroph clones were the outcome of a more or less orthodox sexual mechanism involving zygote formation, crossing over and meiosis. The validity of this assumption was strengthened when Lederberg (1949; Zelle & Lederberg, 1951) described the occurrence of prototroph strains which behaved like heterozygous diploids in continually segregating out different but stable recombination types. As a result of these findings, most published work on recombination in K-12 has been concerned with the elaboration of linkage behaviour and the application of the phenomenon to analysis of such problems as the genetic basis of resistance to antibiotics (e.g. Cavalli & Maccacaro, 1950; Newcombe & Nyholm, 1950*a, b*), while further investigation of the mechanism of recombination itself has been somewhat neglected.

The first indication of sexual differentiation among K-12 mutants was the observation by Hayes (1952*a*) that streptomycin destroyed the fertility of only one of the two equally sensitive mutants with which he worked. He interpreted this differential action of streptomycin as providing evidence against the occurrence of conjugation and as suggesting the uni-directional transfer of genetic material from a 'gene donor' to a 'gene acceptor' cell. He postulated that the living donor cell extruded genetic elements which adhered to its surface so that the cell could continue to function as a gene carrier despite its subsequent 'killing' by streptomycin. On the other hand, the role of the acceptor cell was the vital one of taking up genes from the donor cell and incorporating them into its genetic structure. Hayes (1952*b*) later showed that exposure of his donor strain to small doses of ultraviolet light increased its fertility fivefold or more, while similar treatment of the acceptor strain reduced fertility *pari passu* with the viable count. Moreover, the conditions necessary for ultraviolet enhancement of donor cell fertility closely paralleled those described by Lwoff and others (Lwoff, Siminovitch & Kjølgaard, 1950*a, b*; Lwoff, 1951) for the maturation of prophage and subsequent liberation of lytic phage from lysogenic bacteria. Since both K-12 mutants carried

λ phage (Weigle & Delbrück, 1951), which has been excluded as a possible agent of recombination (Lederberg, personal correspondence; Lederberg *et al.* 1952), it was undecided whether the effect of ultraviolet irradiation on donor cell fertility was primary or merely secondary to λ release, as suggested to the author by Lederberg.

Confirmation of sexual differentiation between K-12 mutants has been reported by Lederberg *et al.* (1952), whose findings are essentially similar to those described below. This paper concerns an investigation into the cause of infertility arising spontaneously in a pair of K-12 mutants which had previously shown normal recombination.

MATERIALS AND METHODS

Bacterial strains

(a) K-12 (wild-type strain). This strain is prototrophic and grows well in unsupplemented minimal medium.

(b) 58-161. A methionine-requiring (M⁻) mutant of K-12 which ferments lactose, maltose, mannitol, galactose, xylose and arabinose, is sensitive to coliphage T₁ and resistant to T₃ (Lac + Mal + Mann + Gal + Xyl + Arab + T₁^sT₃^r). This mutant was originally described as biotin-dependent as well as M⁻ (Lederberg, 1947). Since, however, it grows optimally in minimal medium + methionine alone, and in which glucose is substituted by an acid hydrolysate of pure sucrose (kindly supplied by Thomas Kerfoot and Co., Vale of Bardsley, Lancashire) it must be presumed not to require biotin now. Despite this, its rate of back-mutation to prototrophism is, fortunately, extremely low, and the development of M⁺ colonies has never been observed from control platings on minimal agar under the conditions employed in recombination tests.

(c) W-677. A mutant of K-12 requiring threonine, leucine and thiamine (vitamin B₁) (TLB₁⁻). Its marker characters are complementary to those of 58-161 (i.e. Lac⁻ Mal⁻ Mann⁻ Gal⁻ Xyl⁻ Arab⁻ T₁^rT₃^s).

(d) Streptomycin (SM)-resistant mutants (58-161/S^r, W-677/S^r etc.) were selected by plating very large inocula of each strain on nutrient agar containing 200–250 μ g. SM/ml. The largest and most rapidly growing colonies were picked, purified by replating on SM-agar and tested for growth on agar without SM. Only one S^r mutant of each strain was used throughout this work. The nutritional and marker characters of these S^r mutants were identical with those of their parent strains.

Stock cultures were maintained on Dorset's egg medium at 4° whence subcultures were made to nutrient broth as required.

Media. The constitution of minimal agar (MA) was that recommended by Tatum & Lederberg (1947) except that asparagine was omitted. Except when otherwise stated, this medium was supplemented with thiamine (MA + B₁) in a final concentration of 0.0005 % (w/v). The term 'prototroph' should properly be reserved for those recombinants having the nutritional independence of the wild-type. Although the majority of recombinants arising on MA + B₁ are

B₁ -, the word 'prototroph' will, for convenience, still be applied to all colonies developing on this medium.

Nutrient broth and *nutrient agar* were those routinely employed by this department and were prepared from a tryptic digest of beef. Whenever possible, the same batch of medium was used over long periods and always throughout one experiment.

Technique of recombination tests

(1) *Standard technique.* Bottles of nutrient broth at 37° were seeded separately with 1/10 vol. overnight broth culture of each of the two strains to be mated. After 3-5 hr. growth at 37°, 5 ml. of each culture were mixed, centrifuged at once, and the deposit washed in three changes of 0.9 % (w/v) NaCl buffered at pH 7.2 (hereafter referred to as saline) and resuspended in 1.0 ml. saline. A standard loopful (i.e. a fully charged 2 mm. diameter welded platinum loop = c. 0.01 ml.) of this suspension was transferred to the surface of MA + B₁ in 3 cm. diameter plates and uniformly distributed with a small glass spreader. Tests involving counts of prototroph colonies were always duplicated or triplicated and the counts made after 40-45 hr. at 37°. Using this technique, the usual 58-161 × W-677 mating yielded 20-80 prototroph colonies per plate, depending on the age of the broth cultures employed. With less efficient mating systems the mixture, after washing, was resuspended in appropriate smaller volumes of saline.

(2) *Simplified screening technique.* Later in this work the need arose for a technique whereby large numbers of colonies could be tested rapidly for capacity to show recombination with a gene acceptor (indicator) strain. A portion of each colony to be tested was picked with a platinum loop and rubbed over an area c. 0.75 cm. diameter on the surface of a nutrient agar plate. About thirty such areas could be accommodated on a 9 cm. diameter plate. After 1-1.5 hr. at 37° the plate was exposed to a standard dose of ultraviolet light and reincubated for 1 hr. The growth on each area was then thoroughly rubbed up in a standard (2 mm.) loopful of a young broth culture of the indicator strain and the plate reincubated at 37° overnight. The mixed growth from each area was suspended in 0.5 ml. saline and a standard loopful of this suspension spread over an area of similar size on MA + B₁. Recombination was assessed by the presence or absence of colonies after c. 42 hr. at 37°. The small amount of nutrient material in the dense saline suspensions usually initiated considerable confluent syntrophic growth but this did not seriously interfere with the reading of results. When the colonies to be tested were S^s, an S^r indicator strain was employed and SM incorporated in the MA + B₁. This effectively abolished syntrophic growth without affecting the development of prototroph colonies. Falsely positive results do not arise with this screening technique, while false negatives never exceeded 10 % and were usually considerably lower. Cultures of all colonies showing aberrant or unexpected results were checked by the standard technique after exposure to ultraviolet irradiation.

Total viable counts. A 1/10⁵ (or other appropriate) dilution of each broth

culture was made just before mixing, and standard (2 mm.) loopfuls spread on nutrient agar in triplicate. Colonies were counted after 16–18 hr. at 30°. Since the strains of bacteria used were 'rough' (with one exception mentioned below), loss of cells during washing was negligible, so that results of viable counts indicated fairly accurately the actual numbers of cells of each mutant participating in recombination.

Technique for determining marker characters of prototrophs

The surfaces of prototroph colonies on MA + B₁ were touched with a sterile wire which was then rubbed over nutrient agar slants in such a way as to yield isolated colonies. After incubation a well separated colony of each isolate was picked and suspended in a small volume of saline to yield a faint turbidity. Using a straight wire, a very small inoculum from these suspensions was spotted to points on the surface of a series of solid indicator media in 9 cm. diameter plates positioned over a template. The following media were used:

(1) *Fermentative capacity.* Peptone water-agar containing 1.0% (w/v) of the carbohydrate + neutral red as indicator.

(2) *Phage resistance or sensitivity.* Nutrient agar plates flooded with undiluted phage suspension containing not less than 10⁹ particles/ml., the excess fluid being withdrawn and the plates dried.

(3) *Streptomycin sensitivity or resistance.* Nutrient agar containing 200–250 µg./ml. streptomycin (SM). A plate of MA + B₁ was always included in the series as a check on prototrophism.

In some later experiments involving the testing of considerable numbers of prototrophs for fermentative and SM characters only, prototroph colonies were touched with a sterile wire and spotted directly to a series of plates of MA + B₁ containing the test carbohydrates in place of glucose (growth indicating fermentation), and of MA + B₁ + glucose + SM (200 µg./ml.).

RESULTS

A strain of each of the K-12 mutants 58-161 and W-677 which had ceased to yield prototroph colonies in mixed culture after storage for a year on inspissated egg in the refrigerator, were kindly supplied by Dr C. C. Spicer. These strains, which will be referred to initially as 58-161/*sp* and W-677/*sp*, were shown to be identical in their nutritional and marker characters to the fertile strains of the same designation described above.

*Analysis of the infertile 58-161/*sp* × W-677/*sp* mating*

Each strain was crossed with the heterologous fertile mutant. The mating 58-161 × W-677/*sp* yielded about the same number of prototrophs as 58-161 × W-677, while the 58-161/*sp* × W-677 and 58-161/*sp* × W-677/*sp* matings were sterile. The inference that 58-161/*sp* was the defective partner of the infertile combination was confirmed for fifteen colonies from a plating of this strain. In order to demonstrate, in so far as was possible, that the infertility of 58-161/*sp* was complete and not merely minimal, a culture of one

of these colonies was tested for recombination with W-677 after ultraviolet irradiation under optimal conditions. No recombination was observed. Following irradiation, however, strain 58-161/*sp* (like 58-161 and W-677) liberated λ phage so that its infertility was not due to failure of some genetic mechanism potentiated by the lysis accompanying phage release.

Behaviour of infertile 58-161/sp as a gene acceptor

These findings had been predicted by the hypothesis that gene donor cells (58-161) differed from gene acceptor cells (W-677) only in the possession of an agency whereby genes, or groups of genes, could be transferred outside the cell and thence, by contact, to an acceptor cell. For the sake of simple exposition the term 'carrier' will be used to denote this agency without any implications as to its nature or function. It seemed, therefore, that the most likely cause of infertility in a previously fertile combination was loss by the donor partner of its carrier. The cells of such a strain would be similar to acceptor cells so far as their potential for recombination was concerned, and should therefore be capable of mating and forming prototrophs with a donor strain of dissimilar genotype.

A streptomycin-resistant mutant of 58-161/*sp* was selected (58-161/*sp*/S^r) and tested in comparison with W-677/S^r for ability to show recombination with K-12 (wild-type) and with ten prototroph strains derived from a 58-161 \times W-677 cross. The 'SRP' (streptomycin-resistant prototroph) method described by Lederberg (1951) was used, in which a mixture of an S^r mutant of a nutritionally dependent strain (e.g. W-677/S^r) and an S^s prototrophic strain is seeded to MA + SM which prevents growth of both parent strains but allows S^r prototrophic recombinants to develop into colonies. Since the rate of mutation to S^r is about one/10¹⁰ cell generations in *Bact. coli* (Newcombe & Hawirko, 1949) the ratio of mutant to recombinant colonies is insignificant. In the present series of tests, control platings of double inocula of each strain on MA + B₁ + SM yielded no mutant colonies. Strain 58-161/*sp*/S^r proved fertile when mated with K-12 and yielded about the same number of recombinant colonies as the K-12 \times W-677/S^r cross. Similarly, 58-161/*sp*/S^r was successfully mated with nine of the ten prototroph recombinant strains tested, the one strain which did not form recombinants failing to do so with W-677/S^r also. The findings described above are summarized in Table 1. It was evident from this analysis that 58-161/*sp* had lost its donor properties and had in consequence become an acceptor strain displaying the same degree of fertility as W-677 in matings with K-12 and other prototroph donors.

Lederberg *et al.* (1952), in an independent and closely parallel investigation, have suggested the symbol 'F -' to denote strains which are completely infertile on mating together, and 'F +' to denote strains which form fertile matings with F - as well as with other F + strains. Since it has become quite clear that the term 'donor' is synonymous with 'F +' and 'acceptor' with 'F -', this convenient terminology will be used henceforth in this paper. Thus 58-161 is F +, and W-677 and 58-161/*sp* are F -.

Restoration of 58-161/F - fertility

Working on the assumption that the alteration of 58-161/*sp* from F + to F - was due to loss of a gene carrier, it seemed possible that this strain might be able to reacquire its carrier by infection from an F + strain of similar genotype, and thus be restored to the F + state, in much the same way that lysogenic bacteria which have lost their phage can reacquire it by infection.

Table 1. *Summary of results of various matings relating to fertility analysis of the sterile pair of K-12 mutants 58-161/*sp* and W-677/*sp**

Object of matings	Matings	No. prototroph colonies (Average of duplicates)
Initial analysis	58-161/ <i>sp</i> × W-677/ <i>sp</i>	0
	58-161/ <i>sp</i> × W-677	0
	58-161 × W-677/ <i>sp</i>	109
	58-161 × W-677	71
Confirmation by sensitive ultra-violet technique	58-161/ <i>sp</i> /UV × W-677	0
	58-161 × W-677	52
	58-161/UV × W-677	365
Behaviour of infertile 'donor' strain as a gene acceptor	K-12 × 58-161/ <i>sp</i> /S ^r	144
	K-12 × W-677/S ^r	180
	Prototrophs (S ^a):	
	nos. 1, 2, 3, 4,	× 58-161/ <i>sp</i> /S ^r Numerous, not counted
	6, 7, 8, 9, 10	× W-677/S ^r Numerous, not counted
	Prototroph (S ^a):	
	no. 5	× 58-161/ <i>sp</i> /S ^r 0
		× W-677/S ^r 0

58-161 and W-677 = mutants of fertile combination.

58-161/*sp* and W-677/*sp* = mutants of sterile combination obtained from Dr C. C. Spicer.

/UV = exposed to standard dosage ultraviolet light, followed by incubation in nutrient broth for 60 min. prior to mixing with W-677 culture and washing.

/S^r = streptomycin-resistant mutant.

S^a prototrophs derived from a 58-161 × W-677 mating.

Strain 58-161/S^r/F - was marked by resistance to 0.002M-sodium azide (Az^r) so that it could be selected from mixed culture with a doubly sensitive strain and identified with certainty. To a young broth culture of 58-161/F +, 1/20 vol. of a similar culture of 58-161/S^rAz^r/F - was added and the mixture incubated overnight at 37°. A loopful was plated on nutrient agar + SM. After incubation, twenty-five well isolated colonies were picked, purified by replating on SM-agar and checked for azide resistance. Each of the twenty-five recovered strains was then tested by the screening technique for recombination with W-677/F -. In two separate experiments, 8/25 and 10/25 recovered strains (i.e. an average of 36 %) yielded prototroph colonies and had, therefore, become F +.

F + transfer between strains of dissimilar genotype

The uniquely high rate of F - to F + transformation in 58-161/F - suggested that 58-161/F + might transfer its F + factor to W-677/F - with equal efficiency. An S^rAz^r mutant of W-677/F - was grown in mixed culture

with 58-161/F+ under the same conditions as in the previous experiments. Recovered W-677/S^rAz^r isolates showed a 75% conversion rate to F+ as shown by the ability of the isolates to yield prototrophs with 58-161/F-. Repeated experiments using the same technique, or simply employing fermentation capacity as a marker when the originally F- strain was S^rAz^r, have never shown a conversion rate of less than 40% after overnight mixed culture in nutrient broth.

It was realized that the concept upon which these experiments had been planned, i.e. that the agent responsible for F+ transfer and the carrier concerned in recombination were the same, now seemed to lead to the paradox that the real recombination rate was about a million times greater than the rate demonstrable by the usual technique of prototroph selection. If this was indeed the case, frequent rearrangements of marker and nutritional characters would be expected among F+ isolates from an F- strain converted by an F+ strain of complementary genotype. Twenty-five W-677/F+ isolates, from two separate mixed cultures of W-677/F- and 58-161/F+, were tested for alteration in each of ten characters which distinguish W-677 from 58-161. All the isolates conformed to W-677 phenotype.

Properties of the F+ agent

For technical reasons only a small number of experiments on the character of the F+ agent have as yet been performed. The results of certain of these experiments are sufficiently clear-cut, however, to warrant reporting.

Transmissibility and stability. The F+ agent appears to be indefinitely transmissible through a series of F- strains. For example, in the course of preparing various F+ stocks, the following series of transfers was effected without apparent loss in efficiency of transmission:

58-161/F+ → W-677/S^rAz^r/F- → 58-161/F- → W-677/F- →
58-161/S^rAz^r/F-.

Stocks of F+ strains obtained in this way have proved stable on subculture and on storage for several months on Dorset's egg medium at 4°.

Filterability. The F+ agent appears to be held back by a collodion membrane of 0.74μ. A.P.D. In three experiments, overnight growth of W-677/F- in filtrates of young broth cultures of 58-161/F+ failed to yield any recombinants or any F+ isolates from a total of 115 colonies tested. Even if only a very small number of F+ agents had passed the filter, a disproportionately large number of conversions would be expected from autoinfection during overnight incubation.

F+ transfer in relation to prototroph development on MA. Since all except one of a considerable number of recombinant prototrophs from F+ × F- crosses had proved to be F+, it was decided to determine whether the high efficiency of F- to F+ conversion in nutrient broth obtained also under actual conditions of recombination on MA+B₁. A washed suspension of a mixture of 58-161/F+ and W-677/F- was prepared under standard conditions and three drops (c. 0.06 ml.) spread evenly over the surface of MA+B₁

in a 9 cm. diameter plate. After 24 hr. at 37° developing prototroph colonies were clearly discernible with a hand-lens. Sweeps were made with a wire loop from four areas on the plate (taking care to avoid prototroph colonies), the organisms suspended in saline and then plated on lactose indicator medium. After incubation, 60 W-677 (non-lactose-fermenting) colonies were picked and tested for recombination with 58-161/F⁻. Only 2/60 isolates had become F⁺. On the other hand, when sixty of 102 prototroph colonies which had arisen on the original plate were picked, purified and tested for recombination with 58-161/F⁻ by precisely the same technique as before, 55/60 were found to be F⁺. The remaining five strains which appeared F⁻ proved to be F⁺ also when re-tested by the standard technique following ultraviolet irradiation. Thus, when tested under similar conditions, the F⁺ conversion rate of non-recombinant F⁻ cells under the actual conditions of the recombination test was only 3.6%, while that of recombinants was 100%.

The relative efficiency of F⁺ × F⁻ and F⁺ × F⁺ crosses

The obvious relevance of the F⁺ agent to the recombination process, as shown by the sterility of F⁻ × F⁻ matings, implied that fertility depended either on both mating cells being F⁺, or on one being F⁺ and the other F⁻. If possession of F⁺ by each of two cells is necessary for mating, then clearly an F⁺ × F⁺ cross should be more efficient than an F⁺ × F⁻ one since in the former all the cells are compatible from the start, while in the latter, mating must occur in two stages—first the transference of F⁺ to the F⁻ partner (which was shown above to be inefficient under recombination conditions on MA) and, secondly, the actual mating between the now compatible F⁺ cells. On the other hand, if an F⁺ cell is merely one which possesses a carrier (i.e. is a gene donor) which can only be taken up by a carrier-free F⁻ cell, then an F⁺ × F⁻ cross should be much the more productive. The details of one of several such comparative experiments are given in Table 2.

Table 2. *Relative efficiency of F⁺ × F⁻ and F⁺ × F⁺ matings*

Standard recombination technique employed

Matings	No. prototroph colonies				Av.	Total viable counts (av. of triplicates)	
						Strain	Organisms per ml.
58-161/F ⁺ × W-677/F ⁻	92	105	108	82	97	58-161/F ⁺	945 × 10 ⁶
						58-161/F ⁻	828 × 10 ⁶
58-161/F ⁺ × W-677/F ⁺	5	2	4	5	4	W-677/F ⁺	477 × 10 ⁶
58-161/F ⁻ × W-677/F ⁺	39	59	nt	nt	49	W-677/F ⁻	450 × 10 ⁶

Strain 58-161/F⁺ = original F⁺ strain; strain 58-161/F⁻ = 58-161/*sp.*; strain W-677/F⁺ = W-677/F⁻ converted to F⁺ by 58-161/F⁺.

nt=no test performed.

It will be seen that the 58-161/F⁺ × W-677/F⁻ cross yielded *c.* 20 times as many prototrophs as 58-161/F⁺ × W-677/F⁺. That this result was not due to some abnormality of W-677/F⁺, apart from its acquisition of F⁺, is shown

by the 12-fold rise in recombination rate when this strain was crossed with 58-161/F⁻. Strain 112-12, a non-lysogenic cystine and histidine-requiring F⁺ mutant of K-12 (kindly supplied by Dr A. Lwoff and Dr E. Wollman), was similarly tested against F⁺ and F⁻ strains of 58-161 and W-677. In each case the F⁺ × F⁻ cross was 15-30 times more productive than F⁺ × F⁺.

Strain 58-161/F⁻ is relatively 'smooth' and therefore slower to sediment on centrifugation than the other strains used, so that loss during washing tends to become appreciable. When steps were taken to decrease this loss to a minimum, the matings 58-161/F⁺ × W-677/F⁻ and 58-161/F⁻ × W-677/F⁺ produced the same number of prototrophs on MA + B₁. When, however, samples of these same two mixtures were plated, under the same conditions, on MA alone and on MA + B₁ + T, the relative efficiency of the two matings varied widely, as Table 3 shows. These findings have been confirmed and offer

Table 3. *The effect of growth factor supplements on the relative efficiency of 58-161/F⁺ × W-677/F⁻ and 58-161/F⁻ × W-677/F⁺ matings*

Matings	Standard recombination technique employed		
	Medium		
	MA	MA + B ₁	MA + B ₁ + T
	No. of prototroph colonies (triplicate counts)		
58-161/F ⁺ × W-677/F ⁻	3-2-5 (3)	51-57-54 (54)	113-114-107 (111)
58-161/F ⁻ × W-677/F ⁺	32-35-28 (32)	64-56-58 (59)	22-18-21 (20)
Controls:			
58-161 alone (F ⁺ and F ⁻)	(0)	(0)	(0)
W-677 alone (F ⁺ and F ⁻)	(0)	(0)	(0)

MA = minimal agar; B₁ = thiamine 0.0005 % (w/v); T = threonine 0.0025 % (w/v); strain 58-161 = M - T + L + B₁ +; strain W-677 = M + T - L - B₁ -; () = average of triplicate counts.

some additional evidence for one-way transfer of genetic material from the F⁺ to the F⁻ strain. Thus when W-677, requiring three growth factors (T, L, B₁), is F⁻, the addition of each successive supplement to MA should reduce the number of genetic deficiencies to be made good by transfer from 58-161/F⁺ in order to allow growth, and should therefore increase the apparent recombination rate. This in fact occurs. On the other hand, 58-161 is M⁻ only, so that the efficiency of the W-677/F⁺ × 58-161/F⁻ cross should be unaffected by B₁ and T supplements, except insofar as these supplements might encourage syntrophic growth. Why the addition of threonine decreases the efficiency of this mating is not known, although it may possibly act as a methionine inhibitor. Teas, Horowitz & Fling (1948), working with a doubly deficient *Neurospora* mutant, implicated homoserine as the precursor of both threonine and methionine in this strain and reported inhibition of growth in the presence of excess methionine which was reversed by increasing the threonine concentration.

The relationship of the F⁺ agent to the phenotype of recombinants

Previous analysis of 404 prototroph colonies from sixteen separate 58-161/F⁺ × W-677/F⁻ crosses had shown that, while 279 had a pattern of unselected marker characters (Lac, Mann, T₁ and T₃) different from that of either parent, 103 showed the phenotype of W-677 and only four that of 58-161. Moreover, the four prototrophs displaying the 58-161 phenotype all arose among thirty prototrophs from two consecutive matings. If these two aberrant matings are excluded, then of 374 prototrophs from fourteen separate matings, ninety-seven had the phenotype of W-677 and none that of 58-161. If the two fermentative reactions (Lac, Mann) alone are considered, 62% of prototrophs had the W-677 phenotype of unselected markers. Discovery of the F⁺ factor suggested that this gross bias of recombinants towards the phenotype of the F⁻ (or gene acceptor) strain might be reversed by transposing the F potential of the mating partners. The effect of F⁺ transposition on the phenotype of recombinants is clearly demonstrated by the results of the two experiments presented in Table 4. The number of prototrophs tested is small but the results are clear-cut and reproducible, although

Table 4. *The effect of F⁺ transposition on the phenotype of prototrophs from otherwise similar crosses*

	No. of prototroph colonies having the phenotype of			
	New combinations			
Matings	58-161	W-677		Total
Exp. 1. Marker characters = Lac, Mann, Mal, Gal, SM				
58-161/S ^r /F+ × W-677/F-	0	11	4	15
58-161/S ^r /F- × W-677/F+	11	0	3	14
58-161/S ^r /F+ × W-677/F+	4	6	4	14
Exp. 2. Marker characters = Lac, Mann, phages T ₁ , T ₃				
58-161/F+ × W-677/F-	0	8	2	10
58-161/F- × W-677/F+	13	0	7	20
58-161/F+ × W-677/F+	8	7	5	20
Strain 58-161 = Lac+ Mann+ Mal+ Gal+ phages T ₁ ^s T ₃ ^r				
Strain W-677 = Lac- Mann- Mal- Gal- phages T ₁ ^r T ₃ ^s				

the proportion of prototrophs having new combinations of unselected markers to those having the F⁻ phenotype seems to vary widely from one experiment to another irrespective of the marker pattern used for analysis. Since all these recombinants were obtained by prototroph selection, it was pertinent to determine whether recombinants selected by some other method showed bias of their nutritional requirements towards those of the F⁻ partner. Strain 58-161/F⁺ was mated with W-677/S^r/F⁻ on MA + Lac + SM, supplemented with all the growth factors required by both strains (M, T, L, B₁). Since 58-161 is Lac⁺S^s and W-677 is Lac⁻S^r, only Lac⁺S^r recombinants can develop on this medium. Control experiments showed that no colonies arose from back-mutation when each strain was plated separately. Of forty-

eight recombinant colonies selected in this way, all grew on MA + TLB₁ and only one on MA + M (this isolate also grew on MA alone). Analysis of these strains was restricted by contamination of the available amino-acid preparations by thiamine. However, eleven of twenty-four of these recombinants were found to require T + L for growth and since *c.* 90 % of prototrophs from similar crosses on MA + B₁ are B₁-dependent (Lederberg, 1947) it may be assumed that nearly all of these were TLB₁- and, therefore, of W-677/F- phenotype. The converse of this experiment after F+ transposition has not yet proved possible due to difficulty in obtaining a stable Lac- mutant of 58-161/S^r/F-. It is clear that this dependence of phenotype on the F+ relationships of a mating invalidates the evidence for at least a proportion of genetic linkages previously postulated for K-12. For instance, Newcombe & Nyholm (1950*a, b*) tentatively suggested linkage between S^r and the biotin and methionine loci as the result of reversed crosses in which S^r was introduced alternately into each parent as in matings 1 and 2 in Table 5. Moreover, using 58-161 and W-677, they observed evidence of linkage between S^r and fermentation of the sugars Gal, Mal, Xyl, Arab but not of Lac. Reference to Table 5 shows that when the same reverse crosses are carried out after F+ transposition (matings 3 and 4) the apparent linkage of S^r is the opposite of that implied by matings 1 and 2, indicating that S^r is not linked with methionine. When the sugar

Table 5. *The effect of F+ transposition on the inheritance of streptomycin resistance in reversed crosses*

Matings	No. of prototrophs		No. examined
	S ^r	S ^s	
1. 58-161/S ^r /F+ × W-677/F-	3	149	152
2. 58-161/F+ × W-677/S ^r /F-	146	5	151
3. 58-161/S ^r /F- × W-677/F+	56	8	64
4. 58-161/F- × W-677/S ^r /F+	6	54	60

fermentations of prototrophs from these S^s × S^r crosses are examined the same contrary results are obtained, matings 1 and 2, for example, suggesting positive linkage and matings 3 and 4 negative linkage between S^r and Mal+. Thus the evidence of Tables 4 and 5 invalidates the evidence for the postulated linkages between loci for growth requirements and sugar fermentations, streptomycin and phage susceptibility, and between loci for streptomycin and sugar fermentations.

DISCUSSION

Since all the salient findings described above have been independently discovered and reported by Lederberg *et al.* (1952), and by Cavalli, Lederberg & Lederberg (1953), there can be little doubt as to the validity of the results themselves. Their interpretation, however, is controversial. The chief point at issue, from which any ultimate concept of the physiological and genetic basis of recombination in *Bact. coli* must stem, is whether the phenomenon is fundamentally one of zygote formation followed by crossing-over and segregation

in which the full chromosomal content of each partner participates more or less equally but in which the phenotypic expression of segregants may be profoundly modified by elimination of chromosomal segments introduced by the F^+ partner (Lederberg, 1949; Lederberg *et al.* 1952), or whether there is a one-way transfer of restricted groups of genes from the F^+ to the F^- parent, possibly followed by crossing-over and meiosis limited to the immigrant genes and their alleles, so that the genotype of the progeny remains basically that of the F^- parent (see Hayes, 1952*a, b* and the following discussion).

The concept of one-way gene transfer arose from the observation that SM destroyed the fertility of F^- but not of F^+ cells. The essential validity of this observation was confirmed by Lederberg (personal correspondence) who found that while $S^+F^+ \times S^-F^-$ crosses were productive on SM - minimal agar, the fertility of $S^-F^+ \times S^+F^-$ crosses was negligible. Moreover F^- cells which have been converted to F^+ behave as 'natural' F^+ cells in relation to SM, irrespective of their genotype. There is thus little doubt that SM exerts a differential effect on F^+ and F^- cells. Since both types of cell are equally sensitive to the lethal action of the drug it is logical to conclude that F^+ cells must possess an agent, intimately associated with fertility and at least relatively resistant to the action of SM, which is absent from F^- cells.

The effect of ultraviolet light in stimulating the fertility of F^+ , but not of F^- , cells (Hayes, 1952*b*) confirmed the possession by the former of an additional function related to the recombination process. The similarity of the conditions under which exposure of F^+ cells to ultraviolet became effective, to those required for maturation of prophage in lysogenic bacteria suggested that some agency of the nature of virus or prophage might be the instrument of gene transfer (Lwoff *et al.* 1950*a, b*; Lwoff, 1951). This ultraviolet work has now been in part repeated with each of two non-lysogenic, F^+ mutants of K-12 (kindly supplied by Dr A. Lwoff and Dr E. Wollman, and by Dr J. Lederberg) with the same results, so that the effect of ultraviolet on the recombination potential of F^+ cells is a primary one and not secondary to release of λ phage. Recent work on the action of ultraviolet in inducing synthesis of new substances such as pyocines and colicines (as well as of phage) for which the cells have a covert propensity (Jacob, Siminovitch & Wollman, 1951; Lwoff, 1953), enlarges the field of speculation as to the mechanism of fertility enhancement of F^+ strains of K-12. It was thought not improbable that the agent of genetic transfer, while clearly not λ phage (Lederberg *et al.* 1952), or other potentially lytic phage, might be a non-pathogenic virus whose only overt function was to act as a genetic carrier. The discovery of the F^+ factor with its high efficiency of transfer to F^- cells, its replication within them and its essential role in recombination, appeared to offer strong vindication of this theory. The fact that the great majority of F^- cells, infected with F^+ factor derived from cells of complementary genotype, showed no phenotypic alteration did not exclude F^+ as a genetic carrier, since effective association of such a carrier with genes from the cell it inhabits might well occur only under exceptional physiological conditions. This interpretation appeared the more plausible in view of the

difficulty of accounting for the low rate of recombination among the cells of potentially fertile bacterial clones by more orthodox sexual mechanisms.

The factual relationship of the F^+ factor to recombination is not in dispute: $F^- \times F^-$ matings are completely sterile; maximum fertility is shown by $F^+ \times F^-$ matings, while the fertility of $F^+ \times F^+$ matings is much lower. Lederberg *et al.* (1952) tentatively suggest that this is compatible with a concept of relative sexuality, whereby the fertility of different crosses would be proportional to the F^+ differential between the partners. Thus there would exist different grades of F^+ , F^- having a grade of zero, so that the highest recombination rate would be given by $F4^+ \times F^-$ crosses and progressively lower rates by the series of crosses $F3^+ \times F^-$, $F2^+ \times F^-$ or $F4^+ \times F2^+$, $F4^+ \times F3^+$ and so on. In support of this they show that the ratio of efficiency of $F^+ \times F^+ : F^+ \times F^-$ crosses may vary widely when various combinations of different auxotrophic mutants are tested. On the other hand, Table 3 of the present paper shows that the relative efficiency of $58-161/F^+ \times W-677/F^-$ and $58-161/F^- \times W-677/F^+$ crosses can vary from 1:10 to 5:1 depending on the addition to, or withdrawal from, the minimal medium of growth factor supplements. It is simpler to suppose that fertility depends on the presence of a gene carrier in F^+ cells and its absence from F^- cells. The greater productivity of $F^+ \times F^-$ over $F^+ \times F^+$ crosses is thus explained since in the former every contact between an F^+ and an F^- cell is a potentially fertile mate. The occurrence of limited fertility in $F^+ \times F^+$ crosses requires the assumption that a proportion of F^+ cells from each parental population becomes effectively F^- . It is evident that many F^+ cells must extrude or otherwise liberate their F^+ agent, in order to account for its transmissibility to F^- cells, so that such cells might become effectively F^- until reinfected by F^+ agent from either homologous or heterologous cells. In the latter case a small proportion of reinfected cells would become recombinants. Since in $F^+ \times F^+$ crosses the majority of contacts would be between F^+ cells which form potentially infertile mates, a low degree of fertility would be expected. Direct evidence for the presence of F^- cells in F^+ populations is lacking and would be difficult to obtain since their existence must be transitory due to the efficiency of the F^+ conversion process. All of 140 colonies tested from the plating of a $58-161/F^+$ clone were F^+ . There is, however, some significant indirect evidence. For instance, Table 4 demonstrates that in $F^+ \times F^-$ matings the majority of prototrophs carry the phenotype of the F^- parent and none that of the F^+ parent. In prototrophs from $F^+ \times F^+$ crosses, however, the phenotypes of both parents are more or less equally represented, while the proportion of prototrophs showing new combinations of characters is the same as before. If a proportion of the cells of an F^+ clone are transiently F^- , then treatment of an F^+ culture with SM should destroy the fertility of these F^- cells. Provided that mating can occur only between F^+ and F^- cells, it follows that if only one F^+ parent culture in an $F^+ \times F^+$ cross is treated with SM, then the treated parent should behave as pure F^+ and the untreated parent as pure F^- as judged by analysis of the unselected marker patterns of the

resulting prototrophs. This has recently been demonstrated experimentally, irrespective of which F^+ parent in the cross was subjected to SM treatment. When both F^+ parents were treated with SM, the cross was sterile. Again, if either F^+ parent in an $F^+ \times F^+$ cross is treated with ultraviolet light the prototroph yield increases markedly while the phenotype of prototrophs shifts markedly towards that of the unirradiated strain, i.e. the irradiated parent behaves as if its ' F^+ grade' had been increased. The theory of relative sexuality would therefore predict that if both F^+ parents were irradiated, the prototroph count should fall to its former level from restoration of the relative F^+ grades on both sides of the cross. In fact, when both F^+ parents are irradiated the prototroph count rises to a higher level than when either parent alone is treated with ultraviolet.

The parental phenotypes are not always equally distributed among prototrophs from various $F^+ \times F^+$ crosses, but since in most of these experiments the same F^+ agent was harboured by both parents (as a result of previous direct or serial transmission from one to the other) the variable results would point to different degrees of stability of F^+ in different strains rather than to any qualitative grades of fertility involving all the cells of a strain.

Aberrant linkage behaviour in K-12 has been noted by several workers (Lederberg, 1949, 1950; Newcombe & Nyholm, 1950*a-c*). The attention of Lederberg himself was drawn to these aberrations by the apparent elimination of certain chromosomal segments, involving especially the SM and Mal loci, from persistent K-12 diploids which consequently appeared hemizygous for these characters. It was then found that the segment subject to elimination was that contributed by the F^+ parent. Lederberg *et al.* (1952) therefore tentatively explained the bias of recombinants towards the F^- phenotype by the elimination of segments of the F^+ chromosome after zygote formation. Some of the experimental results recorded here would seem to require elimination of large parts of the F^+ chromosome, or of different parts of it under different environmental conditions of recombination, while the role of F^+ in the matter remains obscure. A more economical hypothesis is that only part of the F^+ chromosome is transmitted to the F^- parent in the first place. If it is supposed, in addition, that the F^+ agent is the genetic carrier, then the determining role of this factor on fertility and phenotype falls neatly into place.

F^+ has many of the characters of a non-lytic infective agent. Since it is hardly to be supposed that its high efficiency of transfer is mediated by cytoplasmic fusion it must presumably leave the cell and, since it is not present in filtrates, remain adsorbed to the cell surface until taken up by an F^- cell. These latter are two of the properties attributed to a hypothetical gene carrier as a result of previous work before the F^+ agent was discovered (Hayes, 1952*a, b*). It was first shown by Cavalli (personal communication) and has been confirmed here and by Lederberg *et al.* (1952) that all recombinant prototrophs (with the single exception mentioned above) are F^+ ; there is also evidence that $F^+ \times F^-$ combinations alone are fertile (so far as individual cells are concerned) and that the direction of gene transfer is from the

F + to the F - parent. Under the actual conditions of recombination in an F + \times F - cross on minimal agar, however, the efficiency of transfer of the F + agent is only 3-4 %. Assuming the premises are valid, there is thus a high degree of probability that the transfer of genes and of F + agent are correlated. Against this may be placed the results of a single experiment which seems to show fairly clearly (1) that while treatment of the F + parent of a cross with ultraviolet light greatly increases the recombination rate, the efficiency of F + transmission is concurrently depressed and (2) that the action of SM on S⁺F + reduces the F + conversion rate markedly in disproportion to the prototroph count when the treated suspension is mixed with an S⁺F - strain. Substantiation of the first of these findings would not invalidate the theory suggested here, for while the F + agent is favoured as the most likely carrier of the genetic elements, the two are regarded as distinct entities. If, for example, one of the effects of ultraviolet light was to increase 20-fold the proportion of F + agents effectively associated with parts of the bacterial chromosome, a concomitant 50 % reduction in the efficiency of F + transfer (approximately that actually observed) would still allow a tenfold enhancement of recombination rate. While discussion of the differential action of SM on the transfer of genes and F + agent is, perhaps, premature, it is possible that an explanation may be found in different degrees of stability of complexes of SM with F + agent alone and with F + agent associated with chromosomal segments, such as have been described for coliphage T₂ and T₄, with and without an external coating of deoxyribonucleic acid, by Cohen (1947*a, b*).

The main aim of this discussion has been to present a theory of recombination which is sufficiently plausible to serve as a useful working hypothesis. It is possible that its substantiation in principle might reveal recombination in *Bact. coli* as a key stage in the evolution of mature sexual processes from simple genetic transformations. The speculative nature of the theory is fully admitted, however, and it is clear that a generally acceptable concept of the intimate mechanism of recombination must await the results of further experimental work.

I wish to express my indebtedness to Dr L. L. Cavalli and Dr J. Lederberg, not only for gifts of cultures but especially for the stimulus which the free exchange of views and experimental results with them has offered me; and to Dr D. A. Mitchison for the benefit of many discussions with him on this subject.

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(Received 7 July 1952)

CAVALLI, L. L., LEDERBERG, J. & LEDERBERG, E. M. (1953). *J. gen. Microbiol.* 8, 89-103.

An Infective Factor Controlling Sex Compatibility in *Bacterium coli*

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SUMMARY: Incompatibility may occur in *Bacterium coli* strains which were previously considered homothallic. A cross between two incompatible strains is completely sterile. Such strains are termed F⁻. Strains which are self-compatible are termed F⁺ and are productive when crossed either with other F⁺ strains or with F⁻ strains. The F⁺ state is transmissible by infection due to a virus-like agent (F) which is not readily separable from the cells. Thus, both in vegetative and sexual reproduction, infection must be mediated by cell to cell contact. No changes other than those of compatibility have been correlated with the F⁺ state. F is independent of λ , the latent phage of the K-12 strain of *Bact. coli*. A small proportion of other strains of *Bact. coli* are fertile when mated with K-12 and, among these, F⁺ and F⁻ strains are found with about equal frequency. In K-12, extreme variations in fertility are found which are only partly associated with F and partly depend on the residual genotype. The cross between two F⁺ strains is usually less fertile than F⁺ \times F⁻ and in such a cross one of the two strains behaves predominantly as F⁻, the other as F⁺. The F⁺ state has a definite effect on segregation in the sense that the genetical contribution of the F⁺ parent to the zygote, or at least to the resulting recombinants, is less than that of the F⁻ parent.

The introduction of methods permitting the selection of rare recombinants from a mass of non-recombinant parental types enabled Lederberg & Tatum (1946) to demonstrate genetic recombination in mixtures of suitably 'marked' mutants derived from the K-12 strain of *Bact. coli*. From the genetic point of view the most typical aspect of recombination in this strain is that exchange of some genetically determined characters involves, at the same time, the exchange of other physiologically unrelated characters with a high probability. Moreover, whole cells have so far been found necessary for recombination to occur, in contrast with transformations or genetic transfers in other bacterial species, such as pneumococcus (Avery, MacLeod & McCarty, 1944; Griffith, 1928; Hotchkiss, 1951), *Haemophilus* (Alexander & Leidy, 1951) and *Salmonella typhimurium* (Zinder & Lederberg, 1952), which have been demonstrated by means of cell-free preparations. Thus, transfer of genetic material in *Bact. coli* K-12 occurs under conditions which imply that some sort of fertilization is taking place, although proof of this must await a morphological demonstration of cell fusion.

Apart from the occurrence of λ phage (see Lederberg & Lederberg, 1953), no infective agent was known to be harboured in *Bact. coli* K-12 until Lederberg, Cavalli & Lederberg (1952) and Hayes (1953) reported the occurrence of a peculiar example of infective inheritance mediated by an agent called F which controls a system of sex compatibility in this strain.

While the original K-12 strain and most of its descendants, obtained by mutation from the wild-type, show no mating limitations, so that this strain was originally described as truly homothallic (Lederberg, 1947; Lederberg, Lederberg, Zinder & Lively, 1951), a few derivative strains have more recently been found which will not cross with one another. These cross-incompatible strains (which are also self-incompatible) have been called F⁻, while strains showing the normal, apparently homothallic condition have been termed F⁺. Experiments have shown that while F⁻ \times F⁻ crosses are completely infertile, F⁻ \times F⁺ and F⁺ \times F⁺ crosses are usually fertile. In practice, the term 'fertility' implies a yield of one recombinant/10⁶ viable parental cells, though the yield is rather lower in the case of F⁺ \times F⁺ crosses and varies with the markers used for selecting recombinants. The term 'incompatibility' means that when c. 10⁹ cells of both parental types are mixed together and plated in media which would normally allow the detection of recombinants, no recombinants arise. Although this, by itself, might merely indicate a frequency of recombination of less than 10⁻⁹, in fact no true recombinants have been isolated from hundreds of crosses between incompatible strains. It is remarkable that one of the standard strains frequently used in crosses, i.e. Y-10 (threonine-, leucine- and thiamine-dependent), as well as all its vegetative descendants, were found to be F⁻, a fact which must be borne in mind when using such strains for genetical experiments. The acquisition of the F⁻ property by Y-10 arose during the second of the three mutational steps whereby this strain was derived from K-12 and has been preserved in several dozen of its descendants throughout innumerable transfers. So far only three other F⁻ clones arising from F⁺ strains, either spontaneously or after mutagenic treatment, have been recorded. A search for F⁻ mutants in F⁺ cultures has proved consistently negative, but the tediousness of the technique has prevented the testing of large numbers of cells.

The finding that all recombinants tested from F⁺ \times F⁻ crosses, irrespective of the type of cross, were F⁺ at once classed the inheritance of F⁺ as different from that of all other markers. Moreover, it was shown that transformation of F⁻ cells into F⁺ could be achieved by contact with F⁺ cells under conditions in which recombination was excluded, as shown by recovery of F⁺ cells having all the marker characters of the F⁻ strain from a mixture of the two types. The term transduction has been employed to indicate this type of transfer of heritable properties (Zinder & Lederberg, 1952), the agent of transfer in the present instance being the infective factor F. The F⁺ property thus acquired by transduction is stable. Loss of newly acquired F⁺ has been noted on only one occasion.

MATERIALS AND METHODS

Crossing techniques. Either of two procedures were used.

(1) Selection of prototrophs (i.e. cells which can form colonies on minimal medium). This method is applicable to the mating of two auxotroph strains, i.e. strains requiring one (monoauxotrophic) or more (polyauxotrophic) growth factors. Fresh overnight nutrient agar slope cultures of the strains to be crossed are harvested in saline, washed repeatedly in saline and 10^8 – 10^9 cells of each strain plated together on a minimal agar medium. Each strain is plated separately as a control, under the same conditions. On the plates spread with the mixture prototroph colonies appear after 24–36 hr. at 37°. No colonies should arise on the control plates. When, as is usual, the number of cells plated of each strain is equal, the yield of recombinants is usually expressed as
$$\frac{\text{no. prototroph colonies}}{\text{total no. cells plated of one parent}}$$

The more commonly used auxotrophic strains were:

Strain 58-161 and its derivatives. These are biotin- and methionine-dependent (BM[−]). The M[−] marker is excellent even when used alone because of its negligible back-mutation rate to M⁺.

Strain Y-10 and its derivatives, requiring threonine, leucine and thiamine (TLB₁[−]).

(2) Selection of streptomycin-resistant prototrophs (SRP selection) (Lederberg, 1951). In this method an auxotrophic streptomycin-resistant strain is crossed to a prototrophic streptomycin-sensitive strain. The mixture of the parents is incubated in broth prior to plating on minimal agar + streptomycin (500–100 µg./ml.). Prior incubation may be omitted if the streptomycin-sensitive (S^a) strain is the F⁺ mate, but even in this case it may increase the yield 100-fold. Time is saved and the prototroph yield increased by aeration of the mixture during incubation in broth. Thus the maximum production of prototrophs was achieved in 4–6 hr. with aeration carried out by rolling, as compared with 12 hr. or more if aeration was omitted.

The differentiation of F⁺ and F[−] strains. To determine whether a strain was F⁺ or F[−] it was crossed with a suitable standard F[−] strain, using the prototroph selection method described above. Since a large number of colonies had to be analysed for F behaviour, the technique was simplified by omitting to wash the strain under test. Two or three loopfuls of growth from an agar slope culture of the test strain ($c. 2 \times 10^9$ cells) were suspended in 0.2 ml. saline. A drop of this suspension was then added to 10^9 washed cells of the standard F[−] strain and the mixture plated on minimal agar. Provided fresh agar slope cultures were used, no interfering background due to residual growth was observed under these conditions. F⁺ strains yielded 200–400 prototrophs while F[−] strains gave none. When the F behaviour of TLB₁[−] strains was under investigation, the standard F[−] strain employed was W-1607 (BM[−]).

Determination of high frequency of recombination (Hfr) behaviour. A derivative of 58-161 was isolated during selection for resistance to nitrogen mustard

which displayed a remarkably high frequency of recombination as compared with standard $M - F + \times TLB_1 - F -$ crosses (Cavalli, 1950). In order to test a strain for Hfr behaviour, 0.05 ml. of suspension of growth from a fresh agar slope culture (*c.* 10^8 cells/ml.) is plated on minimal agar, together with 10^9 washed $TLB_1 -$ cells. An Hfr strain will give 200–400 prototroph colonies under these conditions, while a normal strain of the same biochemical constitution will give not more than one or two colonies.

Unselected fermentation marker characters of prototrophs were tested on EMB medium (Lederberg, 1950*a*; Cavalli, 1950; Lederberg *et al.* 1951).

EXPERIMENTAL

F + transmission

It has been stated in the introduction that $F +$ behaviour, as judged by ability to mate with an $F -$ strain, can be transduced from $F +$ to $F -$ cells by means of an infective process (for details see Lederberg *et al.* 1952). The factor F behaves like a virus in its capacity to infect cells which lack it and to be propagated indefinitely in the new host, but it has no obvious pathological activity.

Since centrifugation removes virtually all transducing activity from the supernatant fluid of $F +$ cultures, it is evident that the majority, at least, of F agents must remain bound to the cells. Cell-free filtrates lend themselves to a more sensitive test of transduction since the $F -$ cells can be left in contact with them for a much longer time and the whole culture subsequently tested for $F +$ behaviour instead of having to rely on the isolation and individual testing of a relatively small number of colonies of the initially $F -$ strain. Despite this, Seitz, Mandler, sintered Pyrex or collodion filtrates of growing and saturated $F +$ broth cultures, of cultures grown in the presence of certain inhibitors (arsenate, citrate, dithionite) of enzymes which might possibly destroy the F agent, penicillin lysates, aqueous extracts of cells ground with alumina and ultrasonic lysates were all ineffective in transduction. It would seem, therefore, that it is the whole cells which are infective. Cells killed with heat (60° for 30 min.) have lost their transducing activity, though a preliminary experiment suggests that this activity may be lost at a proportionately lower rate than viability as judged by colony counts.

While little or no transduction was found to occur in minimal medium, and none in saline or in broth at 4° , when suitable numbers of living $F +$ cells were present under conditions of rapid multiplication in broth, infection is remarkably efficient. For example, if logarithmic phase cells of $F + Lac +$ and $F - Lac -$ are mixed in equal proportions in broth at a concentration of *c.* 5×10^7 cells/ml. and incubated, and at intervals thereafter samples are removed, diluted and plated on EMB-lactose medium so that the originally $F -$ cells can be selected from the mixed culture and tested for $F +$ capacity, it will be found that 50% of the $F -$ cells have been infected after slightly more than 2 hr. incubation. If the same mixed culture is aerated by rolling

which increases the growth rate and, probably more important, the chance of contact between cells, the same result will be achieved in less than 1 hr.

The ratio of F + and F - cells seems to play an essential role in determining the efficiency of transduction. The results of an experiment demonstrating this are given in Table 1. The effect of variation in the F + : F - ratio is clearly seen in the table and is statistically significant. When F + donor cells are in

Table 1. *Effect of varying the ratio of F + to F - cells on the efficiency of F + transduction*

Concentration of F - cells maintained constant. F + and F - cells mixed in the logarithmic phase and culture tubes rolled throughout the experiment. *Medium* = Difco 'Penassay' broth.

Concn. of F - (cells/ml.)	Concn. of F + (cells/ml.)	Ratio, F - : F +	Ratio of no. of F - cells transformed to F + : no. cells tested after		
			$\frac{1}{2}$ hr.	1 hr.	2 hr.
4×10^6	10^6	1:25	20/20	10/10	19/20
4×10^6	4×10^6	1:1	10/20	13/20	6/20
4×10^6	10^6	4:1	2/20	0/19	3/20

large excess, all or almost all F - cells become infected; in general, the fraction of infected cells approximates $(1 - e^{-n_a/n_b})$, n_a being the number of infective and n_b being the number of susceptible cells. This would in fact be the expectation if every F + cell could infect but one F - cell, within the limits of the experiment.

A remarkable feature of this experiment is the lack of correlation between the degree of F + transduction and the duration of contact of F - and F + cells. Such lack of correlation has only been observed in rolled cultures and does not arise in static mixtures. Two possible explanations are (1) that every active F + cell can effect some transfers after which it becomes non-infective for a period of 2 hr. or more, and (2) that the newly infected cells do not transmit F to both daughter cells, thus compensating, at least in a gross way, for increase in frequency of infections with time.

F + and fertility in Bact. coli

The yield of recombinants obtainable from crossing two strains is here called the fertility of the cross. This fertility is dependent on a number of conditions, some of them inherent in the strains, which often make comparison of the fertilities of different crosses impossible. Among the most important of these conditions are:

(a) The genetic linkage relationships of the markers chosen for the selection of recombinants. If such markers are more closely linked, the yield is proportionately lower.

(b) The physiological nature of these markers which may determine the occurrence of a variable degree of syntrophic growth or inhibition of growth, or of growth resulting from the presence of traces of growth factors, when the mixture is plated on minimal agar.

(c) The age of the cultures, those in the logarithmic phase being more fertile than older cultures, both for $F+$ and $F-$ strains.

(d) Whether or not the mixture has been incubated in broth prior to plating. This factor is particularly important when SRP selection is practised.

The effect of extraneous markers can be excluded in comparing the relative fertilities of $F+ \times F+$, $F+ \times F-$ and $F- \times F+$ crosses, however, if $F-$ strains of both parents are available as is the case with $BM-$ and TLB_1- strains. These $F-$ strains can readily be transduced to the $F+$ state, so that $F+$ and $F-$ cultures of both auxotrophs could be used. With these strains the fertility of $F+ \times F+$ crosses was about ten times lower than that of the other two crosses. On crossing another $F+$ auxotroph, W1678, which is proline- and glycine-dependent ($PG-$) with TLB_1- , the $F+ \times F+$ cross was 100 times less fertile than the $F+ \times F-$ cross; unfortunately, an $F-$ strain of $PG-$ was not available for the reverse cross. This relative decrease in fertility was not so marked, however, when $PG-F+$ was mated with $BM-F+$. In some other cases the decrease in the fertility of $F+ \times F+$ crosses was less marked or absent. For example, in the case of TLB_1-S^r and TLB_1+ (the latter obtained by back-mutation from TLB_1-), using strains transduced to $F+$ and the SRP method of prototroph selection, no marked difference was noted between the fertility of $F+ \times F+$, $F+ \times F-$ and $F- \times F+$ crosses. Moreover, crosses between an independent occurrence of $BM-F-$ (strain #8) and TLB_1-F+ and other $F+$ auxotrophs were found to have a very low fertility, thus showing that factors other than F must be taken into account in considering aberrant fertility behaviour.

Temporary phenotypic $F+$ to $F-$ alteration due to environment ($F-$ phenocopy). One other case must be mentioned in which the effect of $F+$ was manifested in a reduced yield from $F+ \times F+$ crosses. This was observed by making use of $BM-F-$ obtained, not by hereditary loss of the F agent, but by temporary suppression of its activity in $BM-F+$ which had been grown to saturation under conditions of aeration, either by rolling or bubbling air through the cultures. Under these special physiological conditions, $BM-F+$ behaves as $F-$, although subsequent growth under ordinary conditions causes a return to the normal $F+$ state. The effect, therefore, is not heritable. Moreover, it is not observed in aerated logarithmic phase cells nor when N_2 or CO_2 are bubbled through the cultures instead of air. It seems likely, though not proven, that a metabolite accumulating in fully grown aerated cultures is involved. This aeration effect is especially marked in strains related to $BM-$, while aerated cultures of other $F+$ strains yield at least some prototrophs when crossed with standard $F-$ strains.

The fertility of Hfr strains. A most interesting variation in fertility has been observed in a strain, isolated from 58-161 after selection for nitrogen mustard resistance (Cavalli, 1950), which showed a remarkably high frequency of recombination (Hfr) when crossed with TLB_1-F- . This Hfr strain cannot be distinguished on morphological or biochemical grounds from the 58-161 ($BM-$) strain from which it was derived and which shows a normal frequency of recombination (Nfr). The Hfr strain is unstable, reverting to Nfr. This

instability has been observed repeatedly at Cambridge and Milan (Cavalli), and at least once at Madison, but the frequency of back-mutation has not yet been established. Hfr seems to be fairly rapidly outgrown in mixed culture with its parent (Nfr) strain, but no infective transfer of Hfr or Nfr behaviour has been noticed under such conditions. Because of the instability of Hfr with reversion to the Nfr state, the strain may be lost unless the isolation and testing of single colonies is carried out at intervals. Moreover, recently isolated subcultures of this strain may be necessary for experimental work as older cultures may contain both the Hfr and the reverted types.

When plated with $TLB_1 - F -$ on minimal agar, Hfr is 100 to 1000 times more fertile than its $BM - F + (Nfr)$ parent strain. Most $Hfr \times F +$ crosses give a prototroph yield lower than $Hfr \times F -$ (though this is not clear-cut with $TLB_1 -$ transduced to $F +$) but usually higher than the equivalent $F + \times F -$ cross. $Hfr \times Hfr$ crosses, employing various auxotrophs (and/or other recombination markers such as drug and virus resistances) from Hfr, give fertilities varying from $1/10^4$ downwards. That the high fertility of Hfr is due to a high frequency of recombination, and not to syntrophic interaction with $TLB_1 -$ strains on minimal agar, has been shown by experiments in which selection for recombinants was carried out by the use of non-nutritional markers (e.g. streptomycin and azide resistance) (Lederberg, 1950*b*). Yields of recombinants 100 times as great as those given by Nfr were obtained.

The relationships between Hfr and $F +$ are not clear. Hfr originated from an $F +$ strain. Nevertheless, Hfr strains do not transduce $F +$ in infection experiments. In conditions under which normal $F +$ strains would give 100 % infections, no infections were ever observed using Hfr strains as $F +$ donors. No transfer of $F +$ has been observed even in recombinants, $Hfr \times F -$ crosses giving only $F -$ recombinants which can, however, be transduced to $F +$. On the other hand, $Hfr \times Hfr$ crosses yield only Hfr recombinants. When Hfr reverts to Nfr it displays the normal $F +$ state as has been shown both by its fertility with $F -$ (several independent reversions of Hfr and its derivatives tested) and by its ability to transduce $F +$ to $F -$ (two presumably independent reversions tested as $F +$ donors). Thus, in spite of its capacity to yield $F +$ after back-mutation to Nfr, Hfr should be classed as $F -$ so far as its activity in infection experiments is concerned, and as strongly $F +$ in relation to its activity in recombination.

Occurrence of the $F +$ agent in Bact. coli strains other than K-12

The occurrence of $F +$ in other strains of *Bact. coli* has not been investigated extensively enough. In the survey by Lederberg (1951), of the capacity of about 2000 *Bact. coli* strains to cross with K-12, the K-12 indicator strain mostly used was $TLB_1 - S^rF -$, so that some potentially fertile strains may have been missed because of their $F -$ state. Nevertheless, this survey yielded over fifty new strains which were fertile when crossed with K-12 strains by the SRP selection method. In seven out of thirty of these strains $F +$ has been detected by transduction to $BM - F -$ or $TLB_1 - F -$. Preliminary results indicate that the number of crossable strains which were missed in

the first survey, because of the use of an F⁻ tester stock, may have been nearly as large as the number found. Some preliminary results seem to prove, however, that the system of compatibilities may be more complex than is indicated simply by the F state of such strains.

Cavalli & Heslot (1949) reported a strain of *Bact. coli* (NCTC 123) which was fertile in crosses with BM-F⁺. The original growth requirements of this strain are complex; good growth can only be obtained with casein hydrolysate, the addition of amino-acid mixtures alone being less satisfactory. Starting from reversions of this original strain which were either fully prototrophic or grew in the presence of methionine + lysine, a series of auxotrophic mutants was made by which it was shown that the strain was self-incompatible. Strain NCTC 123 behaves as F⁻ in crosses with K-12 stocks (with some inconsistencies) and F⁺ can be effectively transduced to it from K-12.

A search for the F agent in several infertile strains of *Bact. coli* and in *Salm. typhimurium* did not reveal the presence of this factor by the criterion of transduction. The strain ATCC 9637 (Davis, 1950) was originally thought to be infertile but was then found to be F⁻. This strain was subsequently shown to be crossable, but with an extremely low frequency of recombination.

F⁺ and segregation

For genetical analysis two types of marker characters are used: (a) fixed or selected markers, such as growth factor requirements or, less frequently, drug resistances, which are used to select recombinants, and (b) markers such as, usually, ability to ferment lactose, galactose, maltose, etc., resistance to viruses and drugs or any other differential character between the strains crossed which are not directly selected in recombination (free or unselected markers). Each marker is obtained by one-step mutation in one of the two strains to be crossed, so that the two strains differ with respect to each marker. The segregation of unselected markers shows well reproducible ratios of the two parental types among recombinants selected on the basis of the fixed markers. This has been taken to demonstrate that reduction occurs soon after fertilization, thus allowing both parental types, as represented by one or more unselected markers, to reappear in the immediate progeny. The diploid state may exceptionally last for a number of generations (see later).

Ratios observed for almost all markers among recombinants differ significantly from 1:1, the ratio to be expected for unselected markers segregating independently of the fixed markers. This forms the experimental basis for linkage. A more refined analysis suggests that all the markers so far employed are linked together, i.e. that they fall into the same 'linkage group'. The evidence for linkage would not be complete, however, on this basis alone. An essential requirement of genetic linkage is that reversal of the segregation ratio is observed when an unselected marker is switched over from one to the other of the two parents (Lederberg, 1947). This type of evidence, which is the distinctive element of Mendelian inheritance, can be expressed in general terms as follows: if, in a cross between two strains A-B⁺C⁺ and A+B-C⁻, recombinants selected for A+B⁺ are found to be p% C⁺ and

(100- p)% C-, then in the reversed cross A-B+C- \times A+B-C+, p % of C- and (100- p)% of C+ recombinants should occur. This has actually been observed, (Lederberg, 1947; Rothfels, 1952; Cavalli, 1952) apart from minor deviations which do not throw any doubt on this major point (Bailey, 1951).

The evidence for linkage suggests that genetic transfer is effected by something more complex than a bag of transforming principles; the contents of the bag must at least be arranged in an orderly way. The next problem, therefore, was to determine the nature of the order. Linear order was assumed by Lederberg on the basis of his early results (1947), but the extension of analysis to other markers (Lederberg, 1949; Newcombe & Nyholm, 1950*a, b*; Cavalli, 1950; Lederberg *et al.* 1951) seemed to show that these later findings could not be reconciled with the original, simple assumption. Among recent contributions, however, that of Rothfels (1952) in favour of linearity may be quoted. It is clear that the problem of linearity will have to be reviewed in the light of the facts given in this paper.

Another, probably related, difficulty arose in the analysis of segregation of diploids. In the case of certain parent strains, known as Het (Lederberg, 1949), some zygotes do not undergo immediate reduction but form relatively stable, persistent heterozygotes which continually segregate out not only the two parental types but also recombinant types, thus showing that crossing-over (assuming this is the genetic basis of the observed exchanges) occurs. Analysis showed, however, that the heterozygotes were not truly diploid in that no segregation of many of the genes closely linked with methionine (e.g. streptomycin, maltose) was observed, so that, with respect to these genes, they carried the gene contribution of one parent only. This is not universal, however, since rare heterozygotes are found from which these genes do segregate (Lederberg *et al.* 1951).

The impact of F+ on the problems of segregation has been twofold. First, it was found that when heterozygotes are formed in a cross between HetF+ and F- strains, it is the contribution from the F+ parent which is partly lost. Such incomplete heterozygotes are thus diploid for a portion of markers contributed by both parents and haploid, or more precisely, hemizygous for the remaining portion contributed by the F- parent alone. The missing portion is always confined to the markers maltose (Mal) and streptomycin (S).

Secondly, it was observed that the pattern of segregation of recombinants is markedly affected by the F types of the parents. This is clearly shown by the data presented in Table 2 which are derived from crosses between TLB₁-S^r and TLB₁+, reversed with respect to the five markers, lactose, galactose, maltose, xylose and arabinose (for which the symbols Lac, Gal, Mal, Xyl, Ara respectively are used). Only the two crosses

Lac - Gal - Mal - Xyl - Ara - \times Lac + Gal + Mal + Xyl + Ara +

and

Lac + Gal + Mal + Xyl + Ara + \times Lac - Gal - Mal - Xyl - Ara -

have been considered out of the possible thirty-two (i.e. 2⁵) crosses which might have been set up because the parents were obtained from a TLB₁-LacGalMalXylAra+ and a TLB₁-LacGalMalXylAra- strain by mutations

for S^r and for prototrophism ($TLB_1 +$) (in three steps). Both these parent strains were F^- so that by transducing each to $F+$, $F+$ and F^- strains of each were available for the examination of all three combinations, $F^- \times F+$, $F+ \times F^-$ and $F+ \times F+$. Since all the marker differences between these strains originated either through ultraviolet induced mutations (for the sugar fermentation deficiencies) or through spontaneous mutations, the chance that chromosome mutations might inadvertently be included, and thus create unwanted complications, is less than if such powerful mutagenic agents as X-rays had been employed. The data in Table 2 comprise the pooled results of a variety of crosses in some of which $F+$ had been transduced to one or other of the parents from different sources (K-12, BM-, W-705). The details of these transductions are irrelevant, however, since $F+$ transductions to the same F^- strain either from different sources or from the same source on different occasions were found to yield the same segregation results. All crosses were carried out by SRP selection after 3 hr. incubation of the mixture in broth in rolled tubes. Some slight heterogeneity was found between identical crosses on different days. This was probably due to lack of close standardization of the age of the cultures to be crossed. In view of this, but more especially because some of the prototroph colonies, which were all scored without isolation, were found to be mixed with respect to some of the sugar markers, a detailed statistical analysis of the data has not been undertaken. In spite of the rawness of the data they provide clear evidence of the effects of $F+$ on segregation. It is noteworthy that the $F+ \times F+$ cross tends to resemble more or less strongly one of the two $F+ \times F^-$ crosses rather than to be intermediate between the two. Numbers are too small, especially in the final column to allow a closer comparison.

The details of a preliminary genetic analysis of these data will not be included here. It may be enough to say that the B_1 marker was found, by crosses not reported here, to fall outside the S^r - TL region, while T and L were found to be closely linked, in agreement with earlier results of the $M^- \times TLB_1^-$ cross (Lederberg, 1947). The five sugar markers (which all belong to strain W-945) seem to be located between S and TL, in the probably linear order: S - Mal - Xyl - Gal - Lac - (Ara - TL). Similar results concerning the effect of F on segregation of prototrophs had been obtained in crosses between BM- and $TLB_1^- F^-$ (parental) or $TLB_1^- F+$ (filial), published by Lederberg *et al.* (1951, table 5), but had, at that time, been interpreted in a different way.

DISCUSSION

Infective inheritance was first described in micro-organisms 25 years ago (Griffith, 1928). It has been found since in a few cases in higher organisms where its role cannot, however, be assessed with certainty. In some species of micro-organisms, e.g. in pneumococcus (Ephrussi-Taylor, 1951; Hotchkiss, 1951) and in *Salm. typhimurium* (Zinder & Lederberg, 1952), infective inheritance seems to have taken over the role of hybridization which is carried out in other organisms through fertilization.

The present case concerns a bacterial species in which genetic transformations induced by means of cell-free preparations have not been satisfactorily demonstrated but in which, on the other hand, a system of genetic transfer by typical hybridization has been described. Oddly enough, the factor showing infective inheritance controls hybridization through control of sex compatibility. Of the several problems which have had to be freshly formulated, or have arisen *de novo*, as a consequence of this finding, none has been solved in an entirely satisfactory way. The first problem is related to the nature of the F agent itself. It behaves like a virus in its capacity to infect without, however, producing any obvious pathological manifestations. The separate occurrence and independent transmission of F and λ phage show beyond doubt that these two agents are quite distinct (Lederberg *et al.* 1952).

The F agent cannot easily be separated from the bacterial cells, but some preliminary experiments suggest that this may eventually be achieved. This difficulty of extracting the virus-like agent or plasmid (Lederberg, 1952) from the cells, or of finding it spontaneously free, may be taken to imply that under natural conditions infection is mostly, or only, due to cell-to-cell contact. This would lend support to the idea that recombinants arise from intimate contacts between cells since all recombinants from $F+ \times F-$ crosses are $F+$. The hypothesis that the F plasmid itself plays a direct role in recombination, being, for instance, the vector of the genetic material which is transferred or an essential part of a 'gamete', cannot be accepted because $Hfr \times F-$ crosses give only $F-$ recombinants. On the other hand, the hypothesis that recombination is the result of some sort of conjugation needs direct, visual support which it is hoped some experiments now planned may supply.

Hfr forms an apparent exception to the rule that, in K-12, the presence of F (i.e. of the $F+$ state) in one of the mates is essential for recombination to occur. This rule would mean that F is, in some direct or indirect way, the determiner of the formation of cells with gametic activities (at least for one sex). For such hypothesis to hold, however, one must be ready to assume that *Hfr* does contain F, though in a bound, non-infective form. That this may be so is implied by the fact, now under closer study, that *Hfr* can revert to $F+$. As to the origin of *Hfr*, the hypothesis that it may be the consequence of mutation of the F agent, resulting in increased gametic activity and loss of infectivity, cannot be discarded, even if it may seem preferable to think that *Hfr* results from a gene mutation at a locus situated in the proximity of markers which are commonly eliminated in crosses with $F-$ strains (to account for the fact that *Hfr* does not reappear from crosses with $F-$). The *Hfr* gene would then exert a control on the activity of the F particles. However, exceptions to the rule that $F+$ is essential for gamete formation may be found in strains of *Bact. coli* other than K-12.

In $F+ \times F-$ crosses, two effects described by Hayes (1952*a, b*) should be taken into consideration; the relative insensitivity to streptomycin of an $F+$ streptomycin-sensitive strain, so far as prototroph forming capacity is concerned, and the ultraviolet stimulation of prototroph formation on $F+$

strains. These effects have probably not been studied extensively enough, and especially in sufficient F^+ and F^- strains, to allow a superimposition of these effects on to the F^+ condition. There may still be room for these effects to be independent of each other, as well as of F , at least to some extent. Within these limitations, the two effects strongly suggest a physiological difference in the gametic activities of F^+ and F^- strains, while the genetical consequence of the F^+ state, as revealed by segregation, demonstrates that the genetic contribution of the F^+ and F^- parent is different. Whether there is also a morphological differentiation between the gametes of an F^+ and an F^- strain in a cross, remains to be determined. If this were so it would imply true sexuality. For the moment it seems valid to retain the use of the term 'sex' in relation to *Bact. coli*, especially in view of the inadequacy of its present definitions.

The role of F^+ in fertility is still less clear. Variations in fertility are found, ranging from the extreme of $F^- \times F^-$ which has zero fertility, to that of $Hfr \times F^-$ which may reach values much higher than the standard rate (i.e. 10^{-6}) of $F^+ \times F^-$ crosses. It seems necessary to assume that residual genotype (i.e. excluding F) plays an important role in fertility. Both the fertility data and the genetical data suggest that in a cross between two F^+ strains, one of the two behaves mostly as F^+ and the other as F^- , the relative role in a cross being determined by the residual genotype. There are some indications that various strains have different F^+ strengths, and that the relative behaviour of the two strains depends on the difference of F^+ strength between the two; the greater the difference the higher the fertility, while the stronger F^+ will behave as F^+ and the weaker as F^- in the cross. The weakest F^+ would be BM^- , with a gradation through TLB_1^- and PS^- to Hfr which would be top F^+ . This hypothesis demands a considerable body of data, both on fertility and on segregation, for experimental analysis. Data based merely on fertility would not seem enough to support or discard it since the hypothesis would not readily yield quantitative predictions of fertility behaviour. An important question would be how much of the fertility in a cross between two strains is predetermined and how much is the consequence of direct interaction between the strains. Experiments testing competitive mating of three or more strains may throw light on this point.

As to the effects of F^+ on segregation, it is obvious that further analyses of linearity of the chromosome (the physical basis of the linkage group) in *Bact. coli* K-12 will have to take them into consideration. At least one hypothesis, based on Mendelian theory, can be put forward to account for them: the elimination of a specific segment of the chromosome contributed by the F^+ parent may take place regularly at every fertilization. There is at present no definite evidence to suggest whether such elimination might occur during formation of the F^+ gametic cell, during fertilization, or at the ensuing reduction.

Another possibility is that there is a different degree of effective ploidy of the F^+ and F^- gametic cells, the F^- gametic cell having a higher degree of ploidy (or, possibly, more nuclei) than the F^+ . In such a case, segregating

markers closely linked with the marker excluded in recombination (e.g. BM -) would have a lower chance of being represented in recombinants when they are carried by the relatively F + parent, than when carried by the F - parent. There is a formal resemblance between this hypothesis and the situation arising in bacteriophage recombination, when a different multiplicity ratio is used for the two parents. This second interpretation, however, does not agree well with some features of the data in Table 2 so that, at the moment, the hypothesis of segmental elimination remains the more attractive.

The work at Madison was supported in part by a research grant (E72-c3) from the National Microbiological Institute, National Institutes of Health, United States Public Health Service, by the Rockefeller Foundation, and by the Research Committee, Graduate School, University of Wisconsin with funds supplied by the Wisconsin Alumni Research Foundation. This constitutes Paper No. 496 in the series of the Genetics Department, University of Wisconsin.

The authors wish to thank Dr W. Hayes for his invaluable help in correcting the manuscript and proofs of this paper.

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(Received 29 July 1952)

The Effect of Serial Passage in other Antibiotics on Penicillinase-producing Staphylococci

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SUMMARY: Seven strains of penicillinase-producing *Staphylococcus aureus* (coagulase-positive) were serially transferred on streptomycin ditchplates; four of the seven streptomycin-resistant variants showed a decreased ability to produce penicillinase. In three instances this loss in penicillinase production was caused by a decreased growth rate. The fourth strain was unstable in relation to penicillin, and exposure to streptomycin appeared to select the naturally occurring penicillin-sensitive variants.

Three of the seven strains were similarly exposed to chloramphenicol; of the three chloramphenicol-resistant variants one was slightly more resistant to penicillin, one considerably less resistant and one unchanged in relation to penicillin. The increased resistance to penicillin was associated with an increase in growth rate. The strain showing a decrease in penicillin-resistance was unstable, and chloramphenicol selected out the naturally occurring penicillin-sensitive variants.

Recent investigations at the Johns Hopkins University (Chandler, Davidson, Long & Monnier, 1951; Monnier & Schoenbach, 1951) have indicated that exposure of penicillin-inactivating bacteria to other antibiotics leads to a reduction in their capacity to produce penicillinase. Monnier & Schoenbach (1951) studied three penicillin-destroying bacteria, one strain each of *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Bacterium aerogenes*. After serial transfer in media containing aureomycin, chloramphenicol, terramycin or streptomycin these organisms showed a 2- to 64-fold increase in sensitivity to penicillin, and this was associated with a loss in penicillinase production. Subsequent transfer of the antibiotic-resistant variants in media free from antibiotics did not lead to a return of their former ability to destroy penicillin. The present investigation was undertaken to study this phenomenon more fully and to see whether it was associated with any other changes.

METHODS

The organisms used were seven strains of coagulase-positive staphylococci, all of which produced penicillinase. Two of the strains (D8 and STH10), both belonging to phage-group 6/47, had been kept in the laboratory for more than a year; five had been recently isolated from the noses of nurses in a maternity department and their phage-types were 3A (strain W187), 3B/3C (strain W120), 47/54 (strain W135R), 52A (strain W305) and non-typable (strain W21).

Serial transfer in the presence of antibiotics was carried out by means of ditchplates. The medium used was 1% 'Lab-Lemco', 1% peptone, 0.5% NaCl and 2% Bacto shred agar. Ditches were cut at one side of the plate

and filled with the same medium containing 100–1000 μ g. streptomycin/ml. or 100 μ g. chloramphenicol/ml. The cultures were plated out at right angles to the ditch and subcultures made from the edge of growth nearest to the ditch. Control cultures were plated on ordinary nutrient agar plates without antibiotic. In the case of streptomycin subcultures were made at 1- to 2-day intervals. With chloramphenicol, at first subcultures were made at similar intervals, but it was later found that resistance to this antibiotic occurred more quickly if the interval was prolonged and subcultures were made after 6–7 days incubation.

Sensitivity to antibiotics was estimated before and after passage in streptomycin or chloramphenicol by the serial dilution method. The medium was Lab-Lemco broth and a large inoculum of 1–6 million bacteria was used. The results were read after 24 hr. incubation at 37°. All the parent strains were inhibited by from 2.5 to 10 μ g. streptomycin/ml., except for strain W305 which was inhibited by 50 μ g./ml., and all were inhibited by from 10 to 25 μ g. chloramphenicol/ml. As regards penicillin, strains D3, STH10 and W21 all grew readily in more than 250 u./ml.; strains W120 and W305 grew in 50 u./ml.; strains W135R and W187 grew in 5–10 u./ml. The penicillin-sensitive strains showed a sensitivity similar to that of the Oxford staphylococcus.

Penicillinase production was measured by the cylinder plate method, using the technique described in a previous paper (Barber, 1947).

Bacteria were counted by the rapid viable counting technique of Miles & Misra (1938).

RESULTS

Exposure to streptomycin

Effect on sensitivity to streptomycin and penicillin. The seven penicillin-destroying strains of *Staph. aureus* were serially subcultured on streptomycin ditch plates. The results of such exposure with regard to streptomycin and penicillin sensitivity are recorded in Table 1. It will be seen that in all strains there was a gross increase in resistance to streptomycin and that four strains

Table 1. *Effect of exposure to streptomycin on sensitivity to (a) streptomycin and (b) penicillin*

Strain	Number of subcultures in streptomycin	Change in resistance	
		Streptomycin increase	Penicillin decrease
D3	12	> 10,000-fold	10-fold
STH10	16	> 10,000	No change
W21, Exp. 1	16	> 10,000	10
W21, Exp. 2	10	4,000	> 1,000
W21, Exp. 3	10	1,000	5
W120	12	> 10,000	No change
W187	7	1,000	2
W305	7	100	2.5
W135R	4	2,000	No change

showed a decrease in resistance to penicillin. This decrease was shown to result from a diminished ability to produce penicillinase.

Effect on growth rate. The four streptomycin-resistant variants with a lower resistance to penicillin than the parent cultures from which they were derived were investigated for other changes. It was found that they also differed from the parent cultures in rate of growth. In strains D3 and W305 the difference was so great that it was readily seen by naked eye inspection of overnight cultures on nutrient agar plates (see Pl. 1, fig. 1). Thus the parent culture showed typical *Staph. aureus* colonies c. 1-2 mm. in diameter, whereas the colonies of the streptomycin-resistant variant were only pin-point in size. When broth cultures inoculated with a known number of organisms were counted at various intervals after incubation it was found, with each of the four strains, that the streptomycin-resistant variant multiplied at a significantly slower rate than the parent culture. The results are given in Table 2, from which it will be seen that the greatest difference in growth rate usually occurred during the first 3 hr. incubation. It seems also certain that, in the case of strains D3, STH10 and W187, the difference in growth rate between parent culture and streptomycin-resistant variant would be quite sufficient to account for a lower rate of penicillinase production leading to the 2- to 10-fold decrease in penicillin-resistance noted with these strains.

Table 2. *Rate of multiplication of parent cultures and streptomycin-resistant variants*

Strain	Increase in population			
	After 3 hr. incubation		After 24 hr. incubation	
	Parent culture subcultured on plain agar	Streptomycin-resistant variant	Parent culture	Streptomycin-resistant variant
D3	15.9 ×	2.3 ×	—	—
W21	19.9 ×	7 ×	170 ×	170 ×
W187	—	—	148 ×	84 ×
W305	19 ×	3 ×	422 ×	244 ×

Stability of strains in relation to penicillin resistance. Many strains of penicillinase-producing staphylococci are known to be unstable in that they yield a proportion of variants which are sensitive to penicillin, having lost their capacity to destroy the antibiotic (Barber, 1949). The incidence of such variants in parent and streptomycin-resistant cultures was investigated. The cultures were plated out thinly on plain agar plates and fifty colonies from each streaked across penicillin ditch plates. The results are given in Table 3.

Strains D3 and W305 did not show penicillin-sensitive colonies, whether or not they had been exposed to streptomycin. Penicillin-sensitive colonies were not isolated from the streptomycin-resistant variant of strain W187, but the parent strain W187 subcultured in the absence of streptomycin yielded three penicillin-sensitive colonies out of fifty tested. Such a proportion of penicillin-sensitive variants, however, would be masked by the penicillinase

of the resistant colonies and have little effect on the penicillin sensitivity of the whole culture (Barber, 1949).

It will be seen that strain W21 is unstable in relation to penicillin, and that after each of three series of transfers in streptomycin the streptomycin-resistant culture yielded a much larger proportion of penicillin-sensitive colonies than did the parent when subcultured in the absence of streptomycin. In Exp. 2, where the streptomycin-resistant variant showed >1000-fold

Table 3. *Incidence of penicillin-sensitive variants after exposure to streptomycin*

No. of penicillin-sensitive variant colonies (fifty tested)

Strain	Parent culture subcultured on plain agar	Streptomycin-resistant variant
D3	0	0
W21, Exp. 1	1	43
W21, Exp. 2	4	50
W21, Exp. 3	2	12
W187	3	0
W305	0	0

decrease in resistance to penicillin, penicillin-resistant colonies were not isolated even when the whole culture was plated out on a penicillin ditch plate. In Exps. 1 and 3, where the decrease in resistance to penicillin was 10- and 5-fold, the streptomycin-resistant variant yielded forty-three and twelve penicillin-sensitive colonies respectively. The cultures of strain W21 not exposed to penicillin only yielded one to four penicillin-sensitive colonies. A typical penicillin ditch plate, showing streaks of penicillin-resistant and sensitive colonies from strain W21, is shown in Pl. 1, fig. 2.

When a penicillin-sensitive and penicillin-resistant colony from the parent culture W21 were serially transferred on streptomycin ditch plates it was found that the penicillin-sensitive variant developed resistance to streptomycin much more quickly than did the penicillin-resistant culture, and after nine subcultures became completely dependent on the antibiotic (see Pl. 1, fig. 3). It thus seemed probable that streptomycin did not increase the incidence of penicillin-sensitive variants from strain W21, but tended to select out those that were naturally occurring.

Stability of streptomycin-resistant variants. Most of the streptomycin-resistant variants remained resistant to streptomycin and showed no change in growth rate after many transfers in broth without antibiotic. The poorly growing streptomycin-resistant variant of strain D3, however, after eight subcultures in the absence of antibiotic, yielded a culture which grew as luxuriantly as the parent strain and showed a 50-fold decrease in resistance to streptomycin. This moderately streptomycin-resistant variant was as resistant to penicillin as the parent culture, although the poorly growing and highly streptomycin-resistant variant had been 10 times less resistant to penicillin.

Exposure to chloramphenicol

Effect on sensitivity to chloramphenicol and penicillin. Three strains were serially subcultured on chloramphenicol ditch plates and then tested for a change in sensitivity to chloramphenicol and penicillin. The results are shown in Table 4. All three strains showed some increase in resistance to chloramphenicol, but the degree was not great. In the case of strain W21, after nine subcultures in chloramphenicol there was a 5-fold decrease in resistance to penicillin and after twelve subcultures the decrease was 1000-fold. In the case of strain W120 there was no change in resistance to penicillin after exposure to chloramphenicol. In the case of strain D3 the chloramphenicol-resistant variant showed a 2-fold increase in resistance to penicillin.

Table 4. *Effect of exposure to chloramphenicol on sensitivity to (a) chloramphenicol and (b) penicillin*

Strain	Number of subcultures in chloramphenicol	Change in resistance	
		Chloramphenicol increase	Penicillin decrease
D3	16	4-fold	2-fold increase
W21	9	2	5
W21	12	10	1000
W120	16	4	No change

Effect on growth rate. The growth rates of the parent and chloramphenicol-resistant cultures in broth were compared after 3–5 hr. incubation at 37°. The results are given in Table 5. It will be seen that the chloramphenicol-resistant variant of strain D3 multiplied somewhat more rapidly than did the parent culture, which probably accounted for the increase in resistance to penicillin. With the other strains there was no significant difference in growth rate between parent and chloramphenicol-resistant cultures.

Table 5. *Rate of multiplication of parent cultures and chloramphenicol-resistant variants*

Strain	Increase in population after 3–5 hr. incubation	
	Parent culture subcultured on plain agar	Chloramphenicol-resistant variant
D3	40 ×	50 ×
W21	17 ×	15 ×
W120	13 ×	11 ×

Stability of strains in relation to penicillin resistance. Fifty colonies from each culture were streaked across penicillin ditch plates. The incidence of penicillin-sensitive variant colonies is recorded in Table 6. Strains D3 and W120 did not yield any penicillin-sensitive colonies, whether or not they had been exposed to chloramphenicol.

Table 6. *Incidence of penicillin-sensitive variants after exposure to chloramphenicol*

Strain	No. of penicillin-sensitive variant colonies (fifty tested)	
	Parent culture subcultured on plain agar	Chloramphenicol-resistant variant
D3	0	0
W21, 9 subcultures	2	46
W21, 12 subcultures	3	50
W120	0	0

Strain W21 was again seen to be unstable in relation to penicillin sensitivity. After nine transfers in the presence of chloramphenicol, forty-six of fifty colonies tested were sensitive to penicillin; after twelve transfers all fifty colonies were penicillin-sensitive, although a few resistant colonies were isolated when a heavy inoculum of the whole culture was plated out on a penicillin ditch plate. After cultivation on nutrient agar without chloramphenicol only two to three penicillin-sensitive colonies were isolated.

DISCUSSION

When penicillinase-producing staphylococci are exposed to streptomycin or chloramphenicol some, but not all, strains show a variable change in their ability to produce penicillinase. The change is usually a decrease, but in one instance was a slight increase. It seems clear, however, from the results here recorded that this is not due to a specific action on penicillinase production, but results from one or both of two associated phenomena. First, the streptomycin- or chloramphenicol-resistant variant may multiply at a different rate from the parent. Secondly, if the original strain is unstable in relation to penicillin, exposure to streptomycin or chloramphenicol may tend to select the naturally occurring penicillin-sensitive variants.

The close association of growth rate with penicillinase production is clearly shown in relation to strain D3. The poorly growing streptomycin-resistant variant of this strain was unstable and reverted to a culture which grew as well as the parent and was only moderately resistant to streptomycin. The parent and the moderately streptomycin-resistant culture were highly and similarly resistant to penicillin, the poorly growing variant showed a 10-fold decrease in resistance to penicillin. Further, when this strain was exposed to chloramphenicol, it yielded a culture which multiplied more rapidly than the parent and showed a slight but definite increase in resistance to penicillin.

The incidence of penicillin-sensitive variant colonies from penicillin-inactivating strains of *Staph. aureus* was studied by Barber (1949) who showed that with some strains variants were few or none, whereas others yield a considerable proportion. Apart from repeated subculture Barber did not find any method of increasing the incidence of these naturally occurring variants. In the present investigation strain W21 was unstable in this respect, and a larger

proportion of penicillin-sensitive variant colonies was isolated after passage in the presence of streptomycin or chloramphenicol than after passage in the absence of antibiotics. When penicillin-sensitive and penicillin-resistant colonies from the parent culture were passaged separately in the presence of streptomycin the penicillin-sensitive culture 'developed' resistance to streptomycin much more quickly than did the penicillin-resistant culture. It seems probable, therefore, that, at any rate with streptomycin, the antibiotic acted by selecting penicillin-sensitive variants, without affecting their actual incidence.

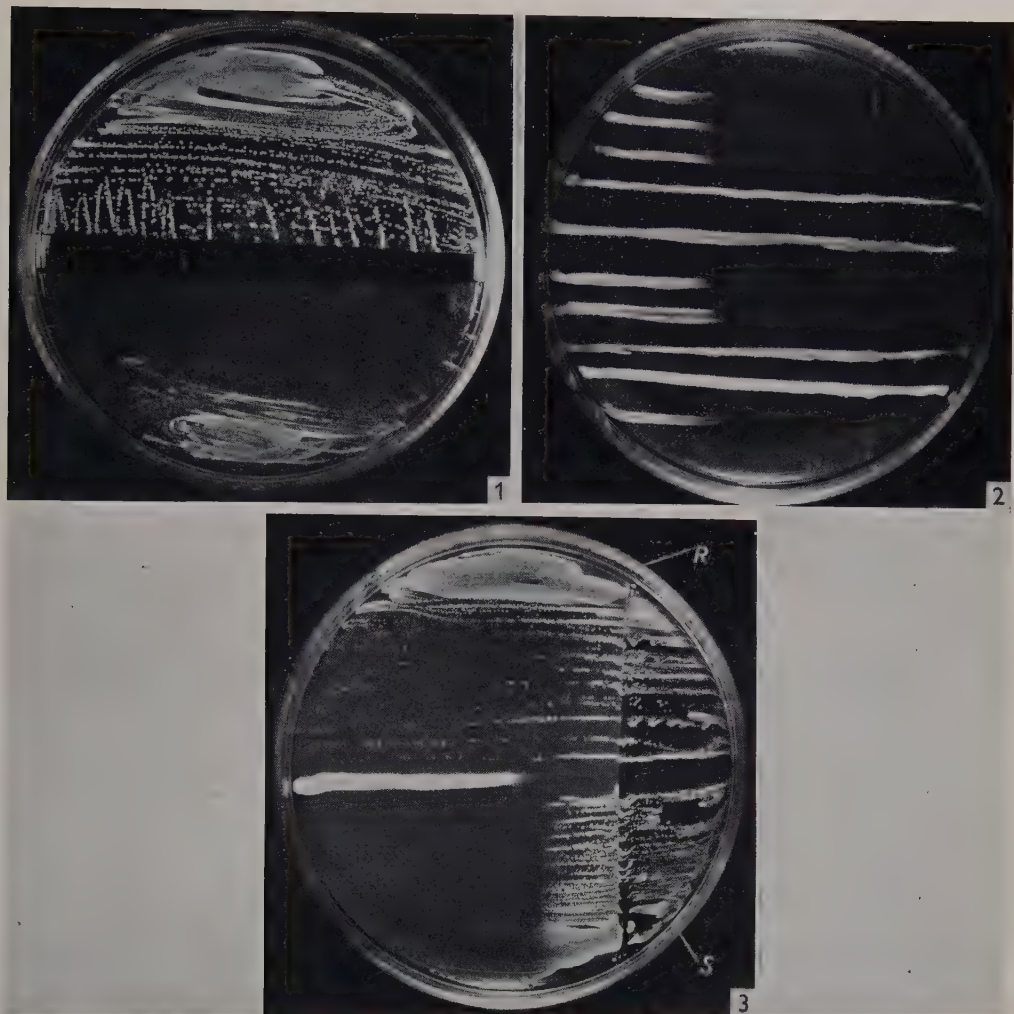
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EXPLANATION OF PLATE

- Fig. 1. Strain D8. Parent culture and streptomycin-resistant variant.
- Fig. 2. Strain W21. Penicillin-sensitive and penicillin-resistant variants streaked across a penicillin ditch plate.
- Fig. 3. Strain W21. Penicillin-resistant (*R*) and penicillin-sensitive (*S*) variants plated out on a streptomycin ditch plate. Penicillin-sensitive culture shows complete dependence on streptomycin and growth of penicillin-resistant culture is favoured by streptomycin.

(Received 9 July 1952)



M. BARBER—PENICILLINASE-PRODUCING STAPHYLOCOCCI. PLATE 1

BARBER, M. (1953). *J. gen. Microbiol.* 8, 111-115.

Penicillin-resistant and Penicillin-dependent Staphylococcal Variants

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SUMMARY: Four strains of *Staphylococcus aureus* (coagulase-positive) were each subjected to three or more series of transfers on penicillin ditch plates. The plates were examined for colonial variants and four quite distinct types of penicillin-resistant variant, one of which was penicillin-dependent, were isolated. These types often occurred in association, and sometimes as many as three were isolated from a single culture plate. The main characteristics of the different types are described.

Studies on the incidence of penicillin-resistant bacterial variants began soon after the isolation of the antibiotic and have continued in increasing numbers (see Abraham *et al.* 1941; Todd, Turner & Drew, 1945; Demerec, 1945, 1948; Bellamy & Klimek, 1948; Gale & Rodwell, 1948; Hughes, 1952). In most studies of this description bacteria have been subcultured in fluid medium containing increasing concentrations of penicillin. In the present investigation solid medium was used to determine whether different types of variant could be isolated from the same culture.

METHODS

Strains of staphylococci. Four strains of *Staph. aureus* were studied; two strains had been isolated from infectious processes and were phage-type 3B/3C (strain D14) and 6/47 (strain STH10) respectively; two strains had been isolated from the noses of nurses in a maternity department and were phage-type 52A (strain M109) and 6/47+ (strain M12).

Penicillin. Crystalline penicillin G (Glaxo) was used throughout.

Passage in the presence of penicillin. The four strains were serially transferred on penicillin ditch plates. The medium used consisted of 1% 'Lab-Lemco', 1% peptone, 0.5% NaCl and 2% Bacto shred agar. Ditches were cut at one side of the plate and filled with the same medium containing at first 10 and later 100 u. penicillin/ml. The ditch plates were stored at room temperature for 24 hr. before use, and subcultures made from the edge of growth nearest to the ditch at 4- to 7-day intervals. Each strain was subjected to three or more such series of transfers.

Sensitivity to penicillin of the parent and variant cultures was estimated by the serial dilution method in 'Lab-Lemco' broth. All the parent cultures were sensitive to from 0.05 to 0.01 u./ml.

Penicillinase production was measured by the cylinder plate method. Young broth cultures of the variants to be tested were mixed with equal quantities of penicillin dissolved in distilled water to give final concentrations of 1-5 u. penicillin/ml. The mixtures were incubated at 37° for 2-24 hr. before transferring to the cylinders.

Inactivation of penicillin. (1) *With penicillinase:* preparation of penicillinase (Burroughs Wellcome), 1 ml. of which inactivated 100,000 u. of penicillin, was used; 0.1 ml. was added to 1000 u. of penicillin in 10 ml. distilled water and the mixture left to stand at room temperature for some hours before use.

(2) *Acid treatment.* Ten ml. 0.01 N-HCl (pH 2.0) was added to an ampoule containing 100,000 u. penicillin and the mixture kept at 37° for 3–6 hr. Tests were carried out with the equivalent of 1 u./ml. penicillin in nutrient agar; at this dilution the acid did not affect the pH of the medium.

RESULTS

Four quite distinct types of penicillin-resistant variant were encountered; they often occurred in association and sometimes as many as three were isolated from a single culture plate. Types 1 and 2 were the most frequently isolated, but, since they grew more luxuriantly than types 3 and 4, this does not necessarily mean that they occurred with greater frequency. An attempt was not made to measure the actual frequency of the variants.

Type 1 variant. This type of variant was isolated from all four strains of *Staph. aureus* and tended to overgrow other types. Its main characteristics are illustrated in Pl. 1, fig. 1. It will be seen that the variant gives typical staphylococcal growth over that part of the plate permitting growth of the parent culture; over the rest of the plate, including the penicillin ditch, growth also occurs, but is less luxuriant and semi-transparent. Gram films taken from opposite halves of the plate also showed a difference. The organisms growing some distance away from the penicillin appeared to be typical staphylococci with a fairly uniform appearance, whereas the organisms growing in the neighbourhood of the penicillin ditch showed gross variations in size and staining, with many large swollen and deeply Gram-positive cells. When subcultures were made on to fresh ditch plates, the same picture resulted, irrespective of which part of the plate the subculture came from.

This type of variant appeared to develop resistance to penicillin gradually and continuously with each transfer, until a culture able to grow in 100 u./ml. or more was obtained. With strain M12, after five transfers a variant of this type appeared which grew to the edge of a ditch containing 10 u./ml., after ten transfers the variant grew right across such a ditch, and after twenty transfers the variant grew right across a ditch containing 100 u./ml. Intermediate degrees of resistance were seen at intervening periods. When such a penicillin-resistant variant was serially transferred in broth without penicillin there was a similarly gradual decrease in resistance, until the culture became as sensitive to penicillin as the parent.

Type 2 variant. Variants of this type occurred very frequently in association with those of type 1. They differed from the latter in that they grew uniformly right across penicillin ditch plates. The degree of growth was variable, but it was usually less luxuriant than that of the parent strains. Gram films showed organisms which were fairly regular in size, shape and staining, but some swollen deeply Gram-positive cells were usually present. Two variants of type 2 are seen in Pl. 1, fig. 2, and variants of types 1 and 2 isolated from

a single culture plate are shown in Pl. 1, fig. 3. Type 2 variants were isolated from strains D14, STH10 and M12. Their origin, degree of resistance and stability were similar to those of type 1.

Type 3 variant. These are small colony variants resembling *g* forms. They showed a slight or moderate increase in resistance to penicillin, which was not increased by further passage in the presence of the antibiotic or decreased by transfer in its absence. This type of variant appeared in a single stage, suggesting a one-step mutation. The variant of this type seen in Pl. 1, fig. 4, was able to grow in a concentration of penicillin sixteen times that which permitted growth of the parent culture. In Gram films of this variant most of the organisms were typical staphylococci, but a few elongated cocci were seen.

Type 4 variant. These variants were found to be penicillin-dependent; maximum growth occurred in the presence of 0.05–1.0 u. penicillin/ml., i.e. about the minimum concentration of penicillin which inhibited the parent cultures. Even in the presence of the optimum concentration of penicillin growth was feeble and on solid medium the colonies were pin-point and semi-transparent. A typical example is shown by itself in Pl. 2, fig. 5, and mixed with the parent culture in Pl. 2, fig. 6. In the latter picture it will be seen that there is a slight gap, containing ghost-like colonies only, between the growths of the parent and variant strains. Gram films of this variant showed a moderate amount of variation in morphology and staining of the cells and some large deeply Gram-positive cocci.

This type of variant was isolated only from strains D14 and M109. In some experiments with these strains, after ten to twenty-five transfers on penicillin ditch plates, all or nearly all the surviving staphylococci appeared to be penicillin-dependent (see Pl. 2, fig. 7). In other experiments variants of this type were selected from plates showing a variety of penicillin-resistant types. Pl. 2, fig. 8, shows variants of types 1 and 4 selected from a single culture plate. The penicillin-dependent variants were extremely unstable, and even in the presence of penicillin tended to yield colonies resembling the parent culture or type 1 variants. Intermediate colonies were not isolated.

Experiments were carried out to determine whether these variants would grow when penicillin was replaced by inactivated preparations. For this, plates were prepared with nutrient agar (1) without addition, (2) +1 u. penicillin/ml., (3) +1 u. penicillin/ml. inactivated with penicillinase, and (4) +1 u. penicillin/ml. inactivated by acid. The results of such an experiment are shown in Pl. 3, fig. 9. The plates were heavily seeded on one half with the parent strains, and on the other half with the penicillin-dependent variant. It will be seen that, apart from the site of heavy inoculum, the variant only grew on the plate containing active penicillin, whereas the parent culture did not grow on this plate, but grew on the other three.

Penicillinase production

Cultures from all four strains were tested for their capacity to produce penicillinase after eight, ten and seventy-five transfers on penicillin ditch plates. Significant destruction of penicillin was not detected. The method

employed, however, was not sufficiently sensitive to detect the destruction of very small amounts, such as may have been utilized by the penicillin-dependent variants.

DISCUSSION

It is clear from these experiments that penicillin-resistant staphylococcal variants may be of many different kinds. Four have been described here, and as many as three were derived from a single strain. Thus mathematical studies, seeking merely to enumerate the cells in a given population able to grow in a given concentration of penicillin, may well be misleading, and can only be used with reserve as a guide to the genetical behaviour of bacteria.

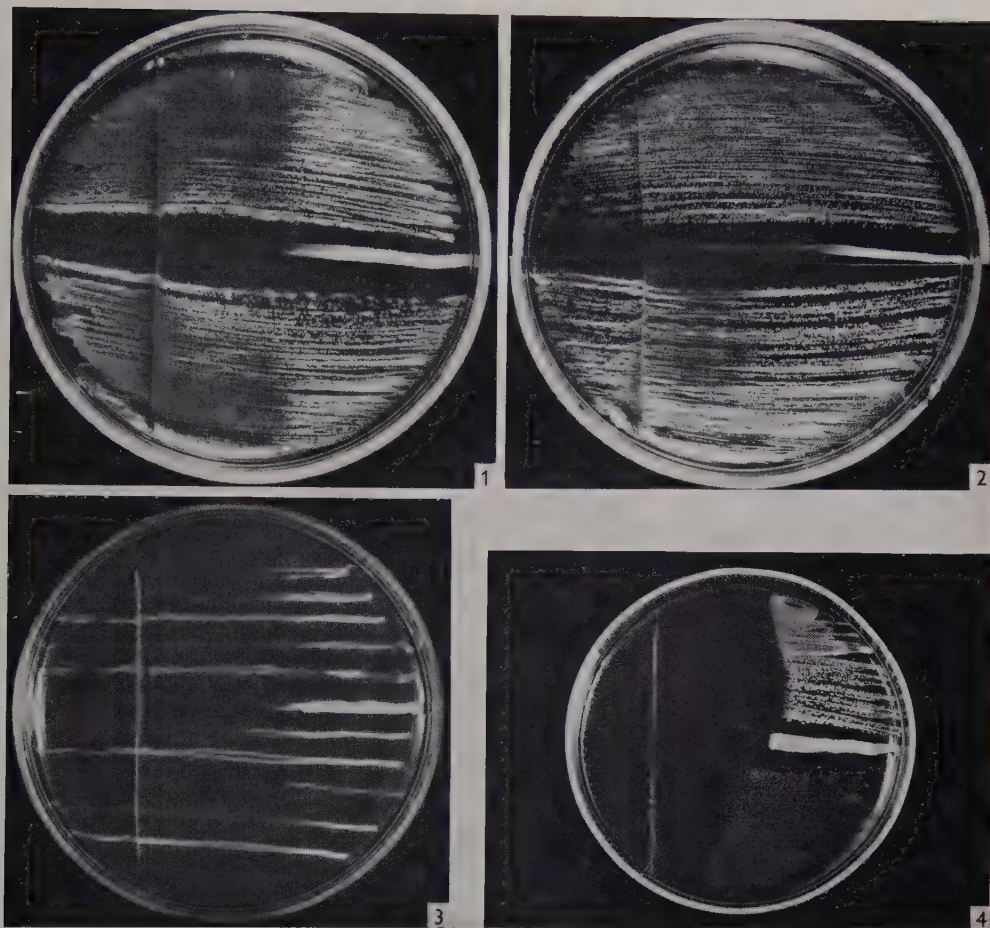
Dogmatic statements cannot be made about the mode of origin of the four types of variant here described. In types 3 and 4, the suddenness of appearance and lack of intermediate types suggests that these variants are mutants. The characteristics of type 1 variants, however, suggest a different mode of origin. With these variants increase in resistance to penicillin occurs gradually, resembling the type of penicillin-resistant staphylococcal variants described by Hughes (1952). Further, these variants lose their increased resistance to penicillin in a gradual fashion when cultivated in the absence of the antibiotic. Both the gain and loss in resistance appear to be a slow continuous change. These features might be compatible with step-wise mutation described by Demerec (1948) but in that case, as Hughes (1952) points out, the number of steps must be very great. Change of an adaptive nature would, therefore, seem a more probable explanation for the emergence of this type of variant.

Nearly all the penicillin-resistant strains of *Staph. aureus* isolated from infective processes are resistant because they produce penicillinase (see Barber, 1949). This type of resistant variant was not encountered in these experiments. Most other investigators have also found that penicillin-resistance acquired by staphylococci *in vitro* is not associated with penicillinase production. Bellamy & Klimek (1948) describe a strain trained *in vitro* to grow in 4 mg. penicillin/ml., which produced small amounts of penicillinase, but only in the presence of penicillin. This strain, however, unlike the penicillin-destroying strains so frequently isolated *in vivo*, had lost many of the properties of a typical staphylococcus. It is unlikely that any of the penicillin-resistant variants here described would be of importance in clinical practice since they would readily be overgrown by typical penicillin-sensitive strains.

My thanks are due to Mr A. L. Wooding for the photographs.

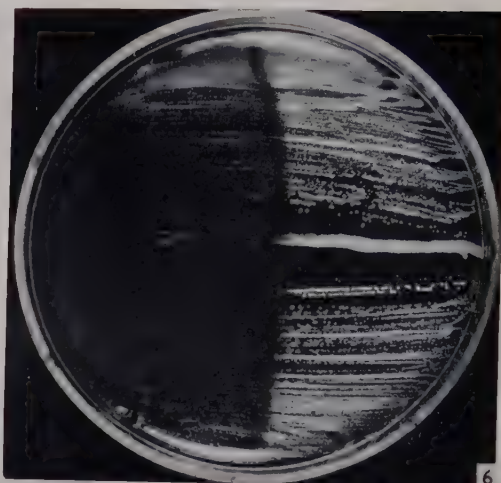
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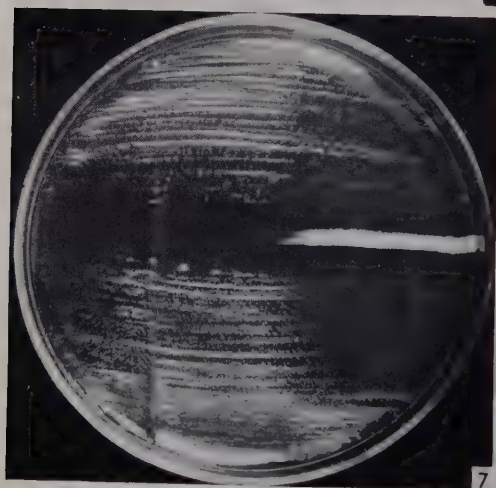




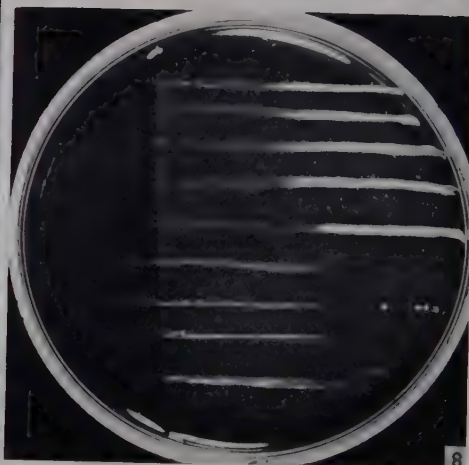
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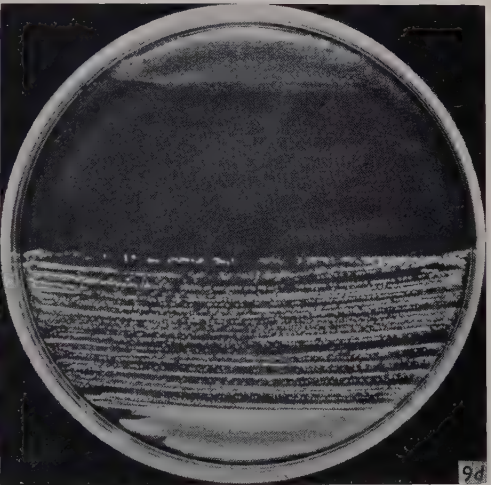
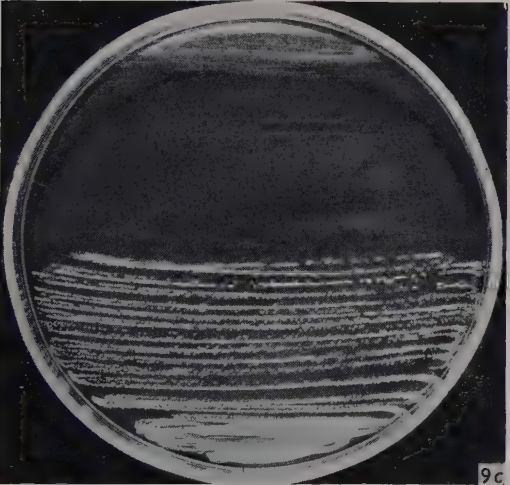
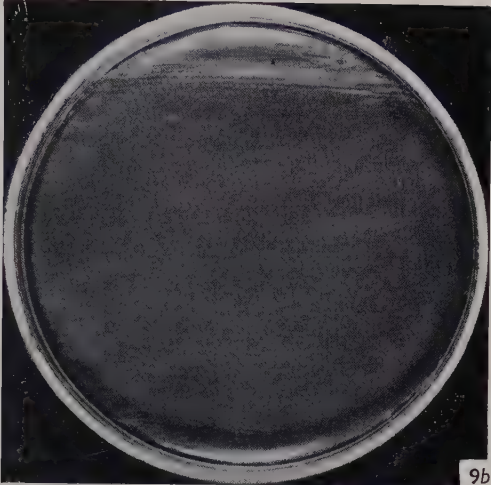
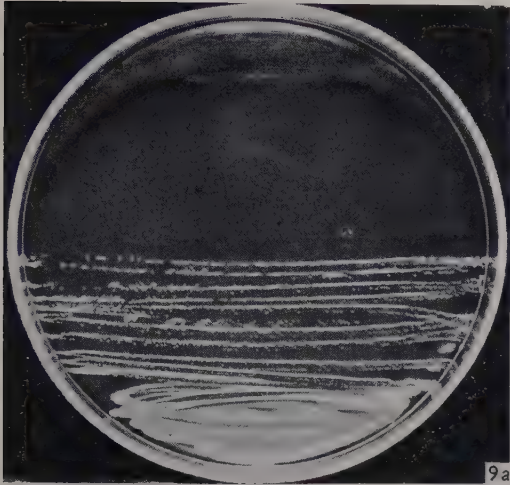
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EXPLANATION OF PLATES 1-3

Figs. 1-8. Penicillin ditch plates; ditch contains 10 u. penicillin/ml.

PLATE 1

- Fig. 1. Type 1 variant derived from strain M12 after eighty-nine transfers. Central streak represents parent culture.
- Fig. 2. Type 2 variants derived from strains STH10 (*a*) and M12 (*b*) after twenty and twenty-five transfers respectively. Central streak represents parent culture of strain STH10.
- Fig. 3. Streaks represent single colonies derived from strain D14 after forty-six transfers. Five streaks represent type 1 variants, four type 2 and one type 3. Central streak represents parent culture.
- Fig. 4. Lower half shows a type 3 variant derived from strain STH10 after twenty-five transfers. Culture on upper half and central streak represent parent culture.

PLATE 2

- Fig. 5. Type 4 variant derived from strain D14 after eleven transfers. Central streak represents parent culture.
- Fig. 6. Same variant as shown in fig. 5 mixed with parent culture. Central streak represents parent culture.
- Fig. 7. Predominance of type 4 variants occurring after strain M109 had been transferred twenty-five times. Central streak represents parent culture.
- Fig. 8. Streaks represent different colonies derived from strain D14 after eleven transfers. Four streaks represent type 1 variants and four type 4. Central streak represents parent culture.

PLATE 3

- Fig. 9. Type 4 variant derived from strain D14 after thirty-four transfers and parent culture plated out on: (*a*) nutrient agar alone; (*b*) nutrient agar + penicillin (1 u./ml.); (*c*) nutrient agar + penicillin (1 u./ml.) inactivated by penicillinase; (*d*) nutrient agar + penicillin (1 u./ml.) treated with HCl at pH 2.0.

(Received 18 July 1952)

The Effect of Physical and Chemical Changes on the Liberation of Phage Particles by Lysogenic Strains of *Salmonella*

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SUMMARY: Phage lysis was induced in lysogenic cultures of *Salmonella* by means of nitrogen mustard, mustard gas, sulphathiazole, glutathione, and sodium thiolacetate.

The liberation of phage particles by these cultures was inhibited by urethane, ascorbic acid, higher concentrations of thiolacetate than that necessary to induce lysis, and by incubating either at 41-43.5° or at pH 5.5. Incubation at 22° in some cases also had a slight inhibitory action.

The following substances had no effect on the liberation of phage particles by these cultures: penicillin, streptomycin, chloramphenicol, Proflavine, Rivanol, colchicine, methionine, cobalt sulphate, sodium citrate, brilliant green, sodium tetrathionate, sodium taurocholate, sodium biselenite, casein digest and yeast extracts. Cultivation either in atmospheres containing different amounts of oxygen, or at 28°, or in media containing varying concentrations of sodium chloride was also without effect.

Cultures did not become non-lysogenic as a result of prolonged passage in broth containing either sodium citrate, ascorbic acid or urethane.

In a previous paper (Williams Smith, 1951) observations were made on the liberation of phage particles by lysogenic strains of *Salmonella thompson*. These could be classified into two types, type 1 in which the number of free phage particles appeared to be directly related to the number of viable bacteria present in the culture, and type 2 in which the number of free phage particles increased more rapidly than the bacteria during the logarithmic phase of bacterial growth, decreased rapidly during a short period at the end of the logarithmic phase and then more slowly. These relations were quite stable when the conditions of experiment were constant. When lysogenic bacteria were actively infected with another phage an increased liberation of particles of the 'lysogenic' phage usually occurred. Since it is generally thought that phages exist within the bacterial cells of lysogenic cultures as non-infective units, I wondered whether active infection had altered the conditions within the bacterial cells permitting the phages to become infective, or whether the phages in these *S. thompson* cultures normally existed within the bacteria as infective units liberated by lysis produced by active infection. It seemed worth while to experiment to find out which was the case, and also to investigate other factors which influence the liberation of phage particles by lysogenic cultures of salmonellas.

That changes can be induced in the host-parasite relationship existing in lysogenic bacteria was first shown by Lwoff, Siminovitch & Kjeldgaard (1950). Working with a lysogenic culture of *Bacillus megaterium* they found

that exposure to ultraviolet light resulted in lysis of the culture with a great increase in the number of phage particles in the culture medium. This observation has been confirmed with other species of bacteria (Jacob, 1950; Cavallo & Cantelmo, 1951; Lwoff & Siminovitch, 1951; Quersin, 1951; Weigle & Delbrück, 1951), although negative results have been obtained with some cultures (Bertani, 1951; Ionesco, 1952). A similar effect to that induced by ultraviolet light has been obtained with X-rays (Latarjet, 1951) and thermal shock (Cavallo, 1951). Phage lysis of lysogenic cultures of *B. megaterium* has also been induced by reducing agents (Lwoff *et al.* 1950; Lwoff & Siminovitch, 1951).

MATERIAL AND METHODS

Basal medium. This consisted of heart broth containing 0.5 % Oxoid peptone and 0.5 % sodium chloride, pH 7.2.

Bacteria. Two of the *S. thompson* cultures had been used in previous experiments (Williams Smith, 1951); they are designated 19 (5/19) and 19 (1/19) and are variants of strain 19 resistant to phage 5/19 and 1/19 respectively. Strain 19 was non-lysogenic and not resistant to any phages active on *S. thompson*.

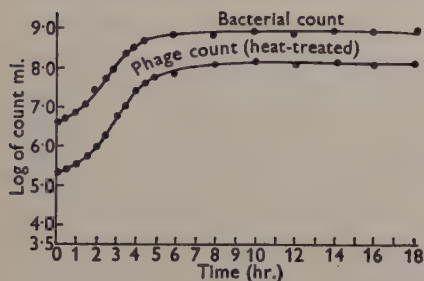


Fig. 1

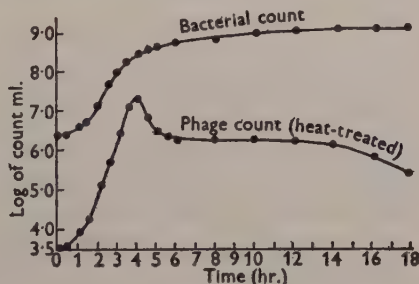


Fig. 2

Fig. 1. The growth rate of bacteria and phage in the lysogenic culture 19 (5/19).

Fig. 2. The growth rate of bacteria and phage in the lysogenic culture 19 (1/19).

The phage/bacterium growth curves of these two strains, which are essentially dissimilar, are illustrated in Figs. 1 and 2. The other lysogenic strains used were naturally occurring ones; these *S. thompson* strains were designated 2, 3, 8 and 11. All six *S. thompson* strains differed from each other either by carrying different phages, or by carrying more than one phage. One of the *S. cholerae-suis* strains was of the *kunzendorf* variety (C11) and the other was the classical species (C16). The *S. typhimurium* strains (M1 and M2) carried different phages.

Bacterial counts. All experiments were carried out in the glass cells of a photo-electric absorptiometer so that the bacterial content could be estimated at any stage of the growth cycle of a culture. However, since it was thought that there might be variation in the size of the bacterial cells, some direct bacterial counts by the method of Miles & Misra (1938) were made in every experiment.

Phage counts. A modification of the Miles & Misra (1938) method for bacterial counts was used for this purpose (Williams Smith, 1951). Nutrient agar (2%) plates were dried for 24 hr. at 37° followed by a further 2 hr. with the lids raised. Four drops (0.08 ml.) of an 18 hr. broth culture of a susceptible strain were spread evenly over the surface of these plates, and drying was continued for another hour with the lids raised. The procedure then adopted was exactly that for bacterial counts. The plates were incubated at 37° for 18 hr. before plaque counts were made.

All lysogenic cultures were heated to 56° for 30 min. referred to as heat treatment, before estimating their content of free phage.

Non-lysogenic strains were used for determining phage counts, strain 19 for *S. thompson* phages, C15 for *S. cholerae-suis* phages, M11 for *S. typhimurium* phages.

Method of carrying out tests. The effects were studied in detail only on the artificially prepared lysogenic *S. thompson* strains 19 (5/19) and 19 (1/19). When a chemical effect was studied varying amounts of the chemical were added to 5 ml. of broth contained in absorptiometer cells. This was inoculated usually with 0.02 ml. of 1/200 dilution of an overnight broth culture of the lysogenic strain. The cultures were incubated at 37°, 0.1 ml. removed at suitable intervals, and its phage content determined. Similar amounts were removed for viable bacterial counts. Phage and bacterial counts on control cultures consisting of the same bacterial strain growing in the basal medium alone were carried out at the same time. Control experiments were also carried out to determine whether the chemical or physical effect under study had any action on free phage particles; reference is made to this type of control in the text only in the mustards since none of the other chemical or physical effects had any observable effect on free phage particles.

The experiments were then repeated with the other lysogenic strains of *S. thompson*, and the *S. cholerae-suis* and *S. typhimurium* strains, but phage and bacterial counts were made only at two stages during the growth cycle.

Some of the chemicals studied caused changes in the medium, as pH value, which were largely incidental to the effect being studied. When possible these incidental changes were corrected and, when possible, the chemical changes being studied were neutralized in the portions of culture medium removed for phage counting.

Growth curves. Many of the results obtained with cultures 19 (5/19) and 19 (1/19) are illustrated by plotting the phage counts against the bacterial counts at different points in the growth cycle. This method has the great advantage of simplicity. Attempts to plot bacterial and phage counts against time of incubation were complicated by effects on the rate of bacterial growth. The time intervals at which counts were carried out were usually farther apart than those shown in Fig. 2, so that the peak of phage production near the end of the logarithmic phase of 19 (1/19) was not always noted.

Cyanide lysis. This was carried out by a modification of Doermann's technique (Rountree, 1951). Overnight broth cultures of lysogenic strains in 10 ml. amounts were centrifuged. The supernatant containing most of the free

phage was discarded and the deposited bacteria resuspended in 2 ml. of sterile distilled water. One half of this was retained at 5° to serve as a control and 0.1 ml. of 0.2M-sodium cyanide added to the other. This was subjected to alternate freezing and thawing until the majority of the bacterial cells had lysed; phage counts were then made. Similar experiments were carried out on the phages carried by the lysogenic strains to determine whether the experimental procedure had any effect on free phage.

The expressions 'lysogenic', 'carrying phage', and 'latently infected with phage' are used synonymously.

RESULTS

The accuracy of the method of counting phage particles

The average of twenty separate counts of the free phage in a culture of 19 (5/19) was 158×10^4 phage particles per ml. with a variation of +13% and -15%.

Cyanide lysis

Cyanide lysis did not result in the liberation of infective phage particles by cultures of the two lysogenic strains 19 (5/19) and 19 (1/19).

The effect of incubation temperature

Growth curves showing the relationship of free phage particles to the number of bacteria in cultures of 19 (5/19) and 19 (1/19) incubated at different temperatures are shown in Fig. 8. No increase in the number of free phage particles in 19 (5/19) was noted at any stage in the growth cycle when incuba-

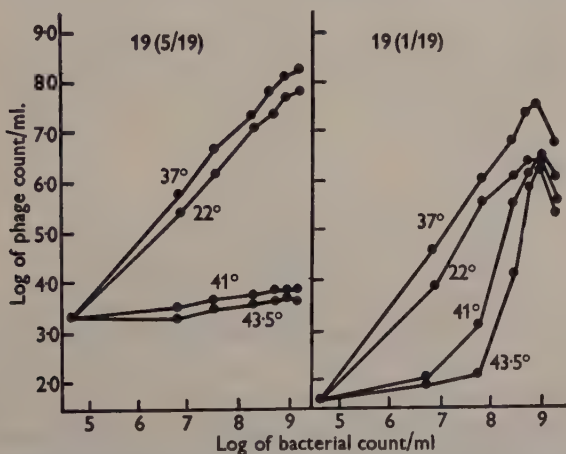


Fig. 3. The effect of incubation temperature on the liberation of phage particles by cultures of 19 (5/19) and 19 (1/19).

tion was carried out at 41 and 43.5°. Very little increase was noted at these temperatures in 19 (1/19) until towards the end of the logarithmic phase when the count increased considerably but not to the level of cultures at 37°. Passage of 19 (1/19) at 43.5° showed a similar curve on each occasion. The

liberation of phage particles was slightly inhibited in both 19 (5/19) and 19 (1/19) when they were incubated at 22°; there was no difference between the results for temperatures of 28 and 37°.

Somewhat similar results were obtained with the naturally occurring lysogenic strains. The results for incubation at 43·5° resembled those for 19 (5/19) more closely than those for 19 (1/19). Greater inhibition of phage liberation was noted at 22° than with 19 (5/19) and 19 (1/19).

Strains 19 (5/19), 2, C11, and C16 were passaged through broth at 43·5° seven times. They were still lysogenic at the final transfer and were liberating little or no free phage.

The effect of hydrogen-ion concentration

Cultures of 19 (5/19) and 19 (1/19) were grown in the basal broth at pH 5·5, 7·2 and 9·0; only at pH 5·5 was any difference noted in the yield of free phage particles (Fig. 4). No difference was noted when the experiment was repeated at pH 6·2. In 19 (5/19) the yield of free phage at pH 5·5 at all stages in the growth cycle was approximately 100 times less than at pH 7·2. Even greater inhibition was noted in the case of 19 (1/19), little phage liberation occurring at pH 5·5.

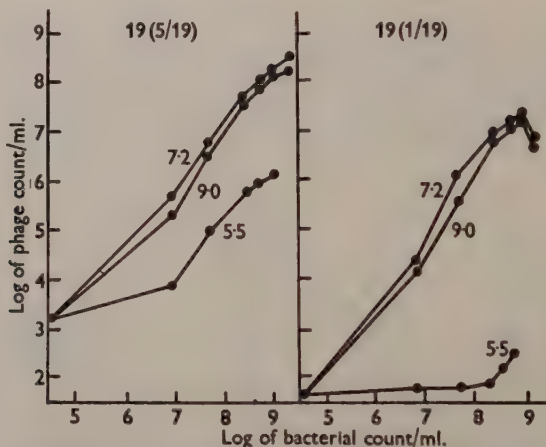


Fig. 4. The effect of pH of the medium on the liberation of phage particles by cultures of 19 (5/19) and 19 (1/19).

The results with five other cultures also showed inhibition of phage production to occur at pH 5·5, the degree of inhibition varying with the culture under test. No phage liberation was detected in one *S. typhimurium* culture at this pH.

When strains 19 (5/19) and 19 (1/19) were passaged three times through broth at pH 5·5 a similar bacterial/free phage growth curve was obtained on each occasion; when passaged strains were then grown in broth at pH 7·2, a normal pH 7·2 growth curve was obtained showing, as in the case of passage at 43·5°, that there was no evidence of adaptation occurring.

The effect of aeration

Bacterium/free phage growth curves of aerated 19 (5/19) and 19 (1/19) were identical with those of control cultures. Aeration had no effect in preventing the phage absorption that occurs at the end of the logarithmic phase of growth of 19 (1/19).

Anaerobic cultivation

When strains 19 (5/19), 19 (1/19), 2, 3, 8, 11, C11, and C16 were grown in the anaerobic atmosphere obtainable in a McIntosh and Fildes jar the amount of free phage liberated was the same as that liberated by control cultures grown in air.

Sodium thiolacetate

The effect of different concentrations of sodium thiolacetate on the liberation of phage particles by 19 (5/19) and 19 (1/19) is shown in Table 1. A great increase in phage particles was noted with concentrations of sodium thiolacetate of between 0.0125 and 0.1 %, the highest yield occurring when the concentration was 0.05 %; concentrations above 0.1 % had an inhibitory effect on free phage production.

Table 1. *The effect of different concentrations of sodium thiolacetate on the liberation of phage particles by cultures of 19 (5/19) and 19 (1/19)*

At the beginning of the experiment 19 (5/19) contained 12.5×10^4 bacteria/ml. and 10^4 phage particles/ml. and 19 (1/19) contained 120×10^3 bacteria/ml. and 10^3 phage particles/ml.

% sodium thiolacetate in medium	Phage particles, 10^4 /ml., liberated at approx. bacterial count of 10^8 bacteria/ml. of	
	19 (5/19)	19 (1/19)
0.2	15	25
0.1	2,500	5,000
0.05	250,000	150,000
0.025	32,000	30,000
0.0125	5,000	2,000
0.00625	1,800	350
0.0	1,550	350

Growth curves of 19 (5/19) and 19 (1/19) grown in broth containing 0.2 and 0.05 % thiolacetate are shown in Fig. 5. Both cultures in 0.05 % thiolacetate broth showed very large increases in the numbers of free particles, particularly in the earlier stages of bacterial growth, the amount of free phage liberated then being about 1000 or more times that found in control cultures. No actual clearing of the broth was noted at this stage but bacterial growth either ceased for a time or proceeded more slowly than in a control. It was difficult to make comparisons on a time basis since thiolacetate normally had a depressant action on the bacterial growth rate. Phage lysis was also induced in the early stages of bacterial growth in media containing 0.2 % thiolacetate. Later, however, this concentration inhibited phage production

and the count decreased, presumably due to bacterial absorption as thiolacetate did not have a lethal action on free phage particles. The process of phage production and absorption fluctuated as shown by the irregularity of the phage/bacterial growth curves.

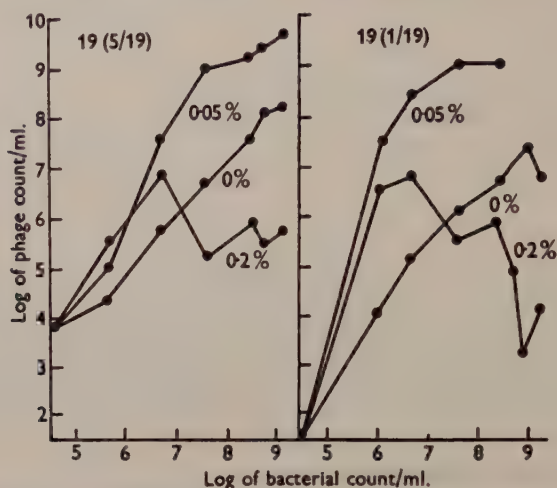


Fig. 5. The effect of the addition of sodium thiolacetate to the medium on the liberation of phage particles by cultures of 19 (5/19) and 19 (1/19).

The results obtained with 19 (5/19) and 19 (1/19) could be repeated at will, and passage of cultures through broth containing thiolacetate showed the same type of free phage/bacterium relation on each occasion. Different concentrations might be required to produce the same effect when different batches of broth were used. The results were also applicable to the naturally occurring lysogenic strains.

In view of Lwoff's (1952) work strain 19 (1/19) was grown in broth containing 0.0005% copper sulphate and different concentrations of sodium thiolacetate, and in a similar series of thiolacetate concentrations with the copper sulphate replaced by 0.0005% cobalt sulphate. The results (Table 2) showed that the yields of free phage at each concentration of thiolacetate were the same whether or not copper and cobalt were added.

Table 2. *The effect of copper and cobalt on the liberation of phage particles by strain 19 (1/19)*

The culture at the beginning of the experiment contained 25×10^4 bacteria/ml. and 10^3 free phage particles/ml.

Media	Phage particles 10^4 /ml., liberated at a bacterial count of 70×10^6 /ml. in media containing sodium thiolacetate in concentrations of					
	0%	0.001%	0.0033%	0.01%	0.033%	0.1%
Broth	360.0	350.0	250.0	350.0	200,000.0	750.0
Broth + 0.0005% CuSO_4	300.0	320.0	350.0	370.0	175,000.0	500.0
Broth + 0.0005% CoSO_4	280.0	200.0	180.0	350.0	150,000.0	600.0

The results of some experiments to elucidate the action of thiolacetate are illustrated in Tables 3 and 4. These consisted of estimating the amount of free phage in cultures of 19 (1/19) in broth containing different amounts of thiolacetate. One culture was aerated, another was in an anaerobic atmosphere and the third had a seal of liquid paraffin. Phage production by means of

Table 3. *The effect of the oxidation/reduction conditions on the induction of phage lysis in a culture of 19 (1/19) by thiolacetate. (I) The effect of aeration and anaerobiosis*

The culture at the beginning of the experiment contained 25×10^8 bacteria/ml. and 10^3 free phage particles/ml.

% sodium thiolacetate in medium	Phage particles, 10^4 /ml., liberated at bacterial count of 40×10^8 /ml. during		
	Aeration	Normal conditions	Anaerobiosis*
0.2	5,000	400	n.t.
0.1	1,000	5,000	2.5
0.04	500	40,000	100
0.02	40	10,000	240
0	200	180	150

* Incubated in McIntosh & Fildes jar.

n.t. = not tested.

Table 4. *The effect of the oxidation/reduction conditions on the induction of phage lysis in a culture of 19 (1/19) by thiolacetate. (II) The effect of a liquid paraffin seal*

The culture at the beginning of the experiment contained 25×10^8 bacteria/ml. and 10^3 free phage particles/ml.

% sodium thiolacetate in medium	Phage particles, 10^4 /ml., liberated at bacterial count of			
	20×10^8		1000×10^8	
	Seal	No seal	Seal	No seal
0.2	150	500	0	0.8
0.1	2,000	25,000	5	50
0.05	60,000	50,000	175	1,500
0.025	20,000	30,000	1,250	12,000
0.0125	300	350	1,750	3,000
0	90	75	400	600

thiolacetate could not be obtained in the anaerobic atmosphere. Only a slight increase in the number of free phage particles was noted in aerated cultures, and then only in broth containing higher concentrations of thiolacetate than induced phage in the control cultures, in fact in concentrations that were inhibitory in controls (Table 3). Bacterial and phage counts were made at two stages in the growth cycle in the paraffin-sealed culture. The results (Table 4) showed that inhibition of phage production, and phage absorption, occurred with lower concentrations of thiolacetate in the paraffin-sealed culture than in the control.

Glutathione

The addition of glutathione to the basal broth induced phage lysis in cultures of 19 (5/19) and 19 (1/19) in a manner closely resembling that produced by thioglycollate, although the effect was not so marked. The most active concentration of glutathione was 0.05 %.

Glucose and iron strips

Growth curves of 19 (5/19) and 19 (1/19) cultivated in basal broth containing different concentrations of glucose and strips of metallic iron closely resembled those obtained in basal broth alone.

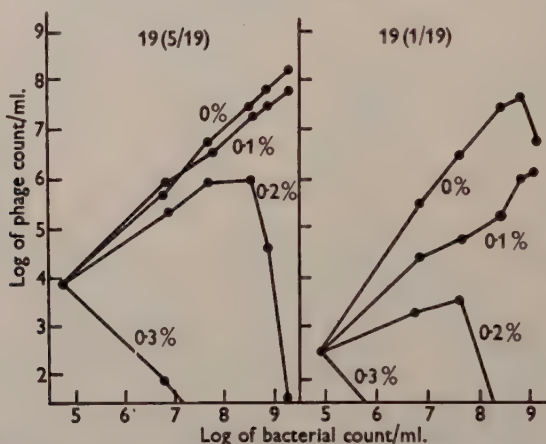


Fig. 6. The effect of the addition of ascorbic acid to the medium on the liberation of phage particles by cultures of 19 (5/19) and 19 (1/19).

Ascorbic acid

Growth curves of 19 (5/19) and 19 (1/19) cultivated in broth containing 0.3, 0.2, 0.1 and 0 % ascorbic acid are shown in Fig. 6. Concentrations of 0.1–0.3 % ascorbic acid had an inhibitory effect on phage production, this effect, aided presumably by bacterial absorption of the free phage particles, being much more noticeable towards the end of the growth cycle, no free phage being demonstrable in cultures grown in 0.2 and 0.3 % ascorbic acid at the end of the cycle. This inhibition was not related to the low pH produced by the higher concentrations of ascorbic acid since acidity was corrected before each experiment. Concentrations below 0.1 % had no effect on phage liberation. Repeated attempts to induce phage lysis by ascorbic acid in a similar manner to thioglycollate were consistently negative. The addition of 0.0005 % copper sulphate to the medium had no effect on the action of ascorbic acid.

The results with other salmonellas were similar to those with 19 (5/19) and 19 (1/19), marked inhibition of phage liberation occurring with concentrations of 0.3 and 0.2 % ascorbic acid and yields similar to those of control

cultures with lower concentrations of ascorbic acid. Strains 19 (5/19) and 8 were passaged through broth containing 0.3% ascorbic acid, each passage taking 10 hr. Single colonies obtained by plating out these cultures after fifty passages were lysogenic.

The effect of sodium chloride concentration

Sodium chloride in final concentrations of 0.5, 1.0, 2.0, 4.0 and 6.0% was added to salt-free broth. With strains 19 (5/19) and 19 (1/19) the yield of free phage was the same irrespective of the sodium chloride concentration. Growth curves of 19 (5/19) and 19 (1/19) in broth containing 0, 0.5 and 4% added sodium chloride were identical as regards phage liberation.

Penicillin

As regards the free phage/bacterium ratio, growth curves of 19 (5/19) and 19 (1/19) in broth containing 8, 10 and 12 u./ml. of penicillin, concentrations which permitted considerable bacterial growth before penicillin lysis took place, were no different from those of controls up to the time when penicillin lysis occurred. Phage counts at different times after penicillin lysis were the same as those before lysis. It was not possible to obtain reliable direct bacterial counts of the broth cultures containing penicillin so, unlike all the other experiments, complete reliance was placed on absorptiometer readings.

The addition of smaller amounts of penicillin to 19 (5/19) and 19 (1/19) so that the bacterial growth rate decreased but no visible penicillin lysis occurred had no effect on the liberation of phage particles.

Phage 5/19 was unaffected by incubation at 37° for 24 hr. in broth containing 1000 u. penicillin/ml.

Streptomycin

Streptomycin in final concentrations of 3–4.5 µg./ml. was sufficient to cause a marked decrease in the growth rate of 19 (5/19) and 19 (1/19), but was without effect on the liberation of phage particles; the free phage/bacterium ratios closely resembled those of control cultures.

Phage 5/19 was unaffected by incubation at 37° for 24 hr. in broth containing 1 mg. streptomycin/ml.

Chloramphenicol

The free phage/bacterium ratio of 19 (5/19) and 19 (1/19) grown in broth containing 1.25–3 µg. chloramphenicol/ml., concentrations sufficient to cause a marked decrease in bacterial growth rate, closely resembled those of control cultures. Phage 5/19 was unaffected by incubation at 37° for 24 hr. in broth containing 1 mg. chloramphenicol/ml.

Sulphathiazole

The effect of the addition of sulphathiazole to the medium on the liberation of free phage particles by cultures 19 (5/19) and 19 (1/19) is illustrated in Fig. 7 which shows that sulphathiazole stimulated phage production so that

at some stages the culture contained 10–500 times that present in control cultures. These experiments were repeated with different concentrations of sulphathiazole and different amounts of bacterial inoculum. The results showed that the amount of free phage liberated was not necessarily directly related to the concentration of sulphathiazole but was dependent on the ability of sulphathiazole to decrease the bacterial growth rate. For example, when broth containing 10 and 100 mg. sulphathiazole/ml. was used (i.e.

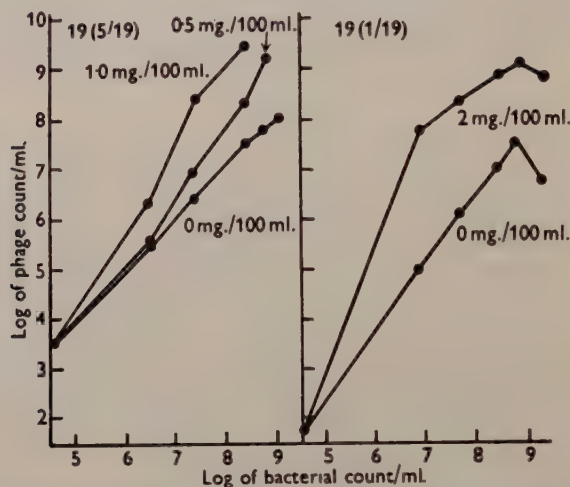


Fig. 7. The effect of the addition of sulphathiazole to the medium on the liberation of phage particles by cultures of 19 (5/19) and 19 (1/19)

concentrations that partially or completely inhibited bacterial growth from the usual small inoculum) and the initial inoculum was sufficiently large to avoid inhibition of the bacterial growth rate, the bacterium/free phage ratio was similar to that of control cultures. Similarly, when the bacteriostatic effect of sulphathiazole was counteracted by *p*-aminobenzoic acid and the usual small inoculum used a normal bacterium/free phage ratio resulted.

When cultures of 19 (5/19) and 19 (1/19) that had been subjected to sulphathiazole were subcultured in the basal broth normal bacterium/free phage ratios were noted showing that sulphathiazole induction was not due to selection of a lytic population of bacteria. Similar results were obtained when sulphamezathine was used instead of sulphathiazole. When the naturally occurring lysogenic salmonellas were grown in basal broth containing sufficient sulphathiazole to decrease the growth rate a considerable increase in free phage usually occurred, although with two strains of *S. thompson*, 2 and 3, no increase was noted and with one of the two strains of *S. typhimurium* the increase was slight.

Proflavine and Rivanol

The addition to the basal broth of 0.00025–0.002 % Proflavine and 0.00025–0.001 % Rivanol had no effect on the liberation of phage particles by 19 (5/19) and 19 (1/19). The higher concentrations were sufficient to decrease the growth rate of both strains.

Nitrogen mustard (di(2-chloroethyl)methylamine)

Preliminary experiments (Table 5) showed that the addition of nitrogen mustard in concentrations of 0.1–0.2 mg./ml. to the basal broth resulted in an increased liberation of phage particles by 19 (1/19). When 0.6 and 0.8 mg./ml. were used no free phage was detected; bacterial growth occurred although, as in the lower concentrations, the growth rate was decreased. Phages 5/19 and 1/19 carried by strains 19 (5/19) and 19 (1/19) were added to broth containing 0.8 mg./ml. of nitrogen mustard and incubated for 1 hr. at 37°. Tests showed that all the phage particles were destroyed, and this lethal effect accounted for the absence of free phage particles in broth containing the higher concentrations of nitrogen mustard.

Table 5. *The effect of different concentrations of nitrogen mustard on the liberation of free phage particles by strain 19 (1/19)*

Each culture at the beginning of the experiment contained *c.* 60×10^8 bacteria/ml. and 10^8 phage particles/ml.

Concentration of nitrogen mustard (mg./ml.)	Free phage particles, $\times 10^4$ /ml., liberated at approx. bacterial count of 300×10^8
0	1,250
0.1	30,000
0.2	10,000
0.4	1,200
0.6	0
0.8	0

The effect of growing 19 (5/19) and 19 (1/19) in broth containing 0.15 mg. nitrogen mustard/ml. is shown in Fig. 8. This shows that at practically all stages in the growth cycle the increase in the liberation of phage particles was between 10 and 100 times greater than that of control cultures. The results of growing other lysogenic salmonellas in broth containing 0.15 mg. nitrogen mustard/ml. showed that in all but one (C16) of the eight cultures a similar increase in the liberation of phage particles occurred.

An alternative method that resulted in a much greater liberation of phage particles was to incubate a culture containing approximately 50×10^8 bacteria/ml. and 0.8 mg. nitrogen mustard/ml. for $\frac{1}{2}$ hr. at 37°. All the free phage was destroyed but the viable bacterial count was unaltered. The culture was centrifuged, the supernatant fluid poured off and the deposit of bacteria mixed with basal broth to the original volume of the culture. This was then

incubated at 37° , together with a control culture not exposed to nitrogen mustard. For a varying time, sometimes up to 2 hr., bacterial growth proceeded at a similar rate in the experimental and control cultures and the same free phage/bacterium ratio existed in each. Growth then ceased in the experimental culture and clearing started. Phage counts carried out during the clearing period showed a great and rapid increase in the number of free phage

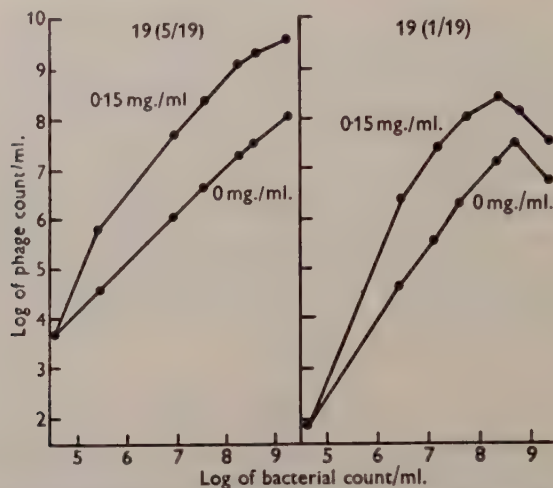


Fig. 8. The effect of the addition of nitrogen mustard to the medium on the liberation of phage particles by cultures of 19 (5/19) and 19 (1/19).

particles. For example, a culture of 19 (5/19) contained 30×10^6 free phage particles/ml. at the stage when bacterial growth ceased and clearing began. Approximately 45 min. later the phage count was 45×10^9 free phage particles/ml. The amount of clearing that occurred varied, but it was never complete. After the stage of clearing, bacterial growth again took place. The results obtained with eight lysogenic cultures are shown in Table 6. In seven very great increases in the liberation of phage particles occurred, but in one, 19 (1/19), despite repeated attempts, it was impossible to induce phage lysis by this method.

Mustard gas

Experiments with mustard gas were carried out with strain 19 (5/19) to see if its action was the same as nitrogen mustard. A small quantity of mustard gas was shaken with the basal broth and allowed to stand for 30 min. The broth was then removed and sufficient 19 (5/19) was added to give a bacterial concentration of approximately 25×10^6 bacteria/ml. Incubation was carried out at 37° for 30 min., the culture centrifuged, the supernatant replaced with an equal volume of basal broth, and incubation continued. Bacterial growth occurred in a similar manner to that obtained with nitrogen mustard. Growth then ceased and lysis occurred with the liberation of large

Table 6. *The induction of phage lysis in lysogenic cultures of salmonellas by exposure to 0.8 mg./ml. of nitrogen mustard for 30 min.*

Culture	Phage particles, 10^4 /ml., liberated	
	After induction	In control culture
19 (5/19)	4,500,000	2,100
19 (1/19)	500	650
2	3,500,000	725
3	3,200,000	2,500
11	1,400,000	825
C16	3,500,000	3,600
M1	2,500,000	8.5
M7	2,100,000	86

The phage counts quoted for the control cultures were those obtained at the stage at which phage induction commenced in the corresponding experimental culture.

Cultures with the prefix 'C' are *S. cholerae-suis* and those with the prefix 'M' are *S. typhimurium*; the others are *S. thompson*.

numbers of phage particles. In one experiment the number of free phage particles/ml. after induction was $75,000 \times 10^5$ compared with 50×10^5 in a control culture. As with nitrogen mustard, mustard gas was more lethal to free phage particles than to bacteria.

Colchicine

Colchicine added to the basal broth in final concentrations of 0.001–0.1 % had no noticeable effect on the bacterial growth rate of 19 (5/19) and 19 (1/19) or on the number of phage particles liberated.

The liberation of phage particles by strains 2, 8 and C16 also was not influenced by 0.1 % colchicine.

Urethane

Urethane in concentrations of 0.03–3 % was without effect on the liberation of phage particles by 19 (5/19) and 19 (1/19) except at the 3 % level when a marked decrease was noted. The higher concentrations had a pronounced inhibitory effect on the bacterial growth rate. Little or no phage was liberated by seven lysogenic salmonellas grown in broth containing 3 % urethane.

Strains 19 (5/19) and 2 were passaged through broth containing 3 % urethane, each passage taking 24 hr. Single colonies obtained by plating out these cultures after fifty passages were all lysogenic.

Sodium citrate

The lysogenic cultures used in the previous experiments were classified by the citrate sensitivity of the phages they carried. Heat-treated 18 hr. broth cultures were titrated for free phage on ordinary nutrient agar with and without 2 % sodium citrate. The phages carried by 19 (1/19), 8, 11 and M1 were completely sensitive and those carried by strains 2, 3, C11 and C16 were completely insensitive. The phages carried by strains 19 (5/19) and M7

were classified as slightly sensitive since the plaques on citrate-agar were less numerous and smaller than those on ordinary agar.

When the strains were grown in basal broth containing 2% sodium citrate there was no difference between the amount of free phage liberated than when they were grown in the basal broth alone. The two strains carrying citrate-sensitive phages, 19 (1/19) and 8, were passaged through broth containing 2% sodium citrate, each passage taking 12–24 hr. Single colonies obtained by plating out these cultures after 100 passages were all lysogenic.

The effect of casein digest, yeast extract, methionine, cobalt sulphate, brilliant green, sodium biselenite, sodium taurocholate and sodium tetrathionate

The following substances when added to the basal broth in the concentrations stated were without any significant effect on the liberation of phage particles by cultures of 19 (5/19) and 19 (1/19):

Substance	Final concentration tested (%)
Casein digest (Bacto-Casitone, Difco)	0.2–1.0
Yeast extract (Oxo)	0.3–3.0
Methionine	0.025–0.2
Cobalt sulphate	0.002–0.008
Brilliant green	0.000125–0.002
Sodium biselenite	0.05–0.2
Sodium taurocholate	0.05–1.0
Sodium tetrathionate	0.1–3.0 (approx.)

DISCUSSION

The results obtained by lysing the bacterial cells of lysogenic strains of *S. thompson* by sodium cyanide and penicillin showed that these cultures resembled other lysogenic cultures; phage was not present within the bacterial cells as infective units capable of producing lysis of susceptible cultures. Williams Smith (1951) observed that it was usually possible to obtain a marked increase in the number of free phage particles liberated by lysogenic cultures of *S. thompson* by producing in them an active infection with another phage. This result was not due to the liberation of phage previously contained within the bacterial cells as infective units. Rather does it suggest that active infection altered the host-parasite relation existing between the bacterial cells and the non-infective phage contained within them thus permitting this phage to become infective and possibly to multiply.

Several views have been put forward to account for the phenomenon of lysogenicity in bacterial cultures. Boyd (1951), working with *S. typhimurium*, considers that phage exists in two forms and that variation may occur from one form to the other. He postulates that the form existing within the bacterial cell, the symbiotic form, on occasion mutates to the lytic form which lyses the bacterial cell and then constitutes the greater part of the phage that is found free in the culture medium of lysogenic cultures. Lwoff & Gutman (1950) brought forward the 'activation' hypothesis. They considered that

the phage existing within the bacterial cell is an incomplete or immature form of lytic phage, called prophage, and that some disturbance of metabolism permits the maturation of some prophage particles, resulting in lysis of the bacterial cell and the presence of free phage in the culture medium. Clarke (1952) and Clarke & Cowles (1952) are of the opinion that bacteriophage normally exists within the bacterial cell as prophage but that occasionally some bacterial cells within a lysogenic culture revert from the resistant to the sensitive state; this allows the prophage to form active or lytic phage which lyses the cell within which it was contained and becomes free in the culture medium. Much of the evidence for this view depended on the observation (Clarke, 1952) that prolonged passage in calcium-free media eventually resulted in a lysogenic culture of *B. megaterium* becoming non-lysogenic and fully sensitive to the phage it carried previously. It is of interest to consider how the results obtained in this present work accord with these views. They show that the free phage/bacterium ratio in lysogenic cultures of the salmonellas studied was normally a constant one, but that it could be upset by many and quite different chemical and physical stimuli resulting either in a great increase or decrease in the amount of free infective phage present in the culture fluid. In some cases it was noted that different concentrations of the same substance, and even the same concentration at different stages during growth of a culture, had quite opposite effects. Again, subcultures of a strain in which free phage production had been induced by, for example, sodium thiolacetate showed a normal free phage/bacterium ratio when grown in ordinary broth or a great increase in the number of free phage particles if grown again in broth containing sodium thiolacetate. Prolonged passage in citrate broth of lysogenic cultures carrying citrate-sensitive phage, or passage in other media which inhibited production of free phage particles, did not produce cultures that were sensitive to the phage which they had previously carried, as Clarke (1952) had done with the strain of *B. megaterium*. These facts do not support the view that lysogenicity is due either to mutation of phage or bacteria if the term mutation is considered in its most commonly accepted meaning. Rather do they support the view of Lwoff & Gutman (1950). It is possible then that the phenomenon of lysogenicity in the cultures examined can be explained as follows. When a susceptible bacterial culture is actively infected with phage, the phage particles, after entering the bacterial cells, split up into a number of sub-units. These multiply and then aggregate into the characteristic pattern of fully formed infective particles of the phage from which they were derived, the bacterial cells lyse, and the infective phage particles become free in the culture medium. In some of the bacterial cells this process does not reach completion and a state of equilibrium is established between phage and bacterial cell. The stage of phage development at which equilibrium is reached would be somewhere between the stage at which multiplication of the sub-units was possible, since bacterial cells resulting from multiplication of latently infected cells contain phage which is able to become infective, and the stage when they coalesce and also become infective. These are the bacterial cells that constitute a

lysogenic culture. The host-parasite relation is not stable; occasionally it is upset in some cells so as to permit the formation of infective phage which lyses the cells and can be demonstrated in the culture medium. Since most studies on bacteriophage have been concerned with the active infection of susceptible bacterial cells, too much emphasis has been placed on this type of relation. It is probable that in nature the normal relation between bacterium and phage is that found in lysogenic cultures and that active infection is relatively a rare event. It might therefore be inadvisable to refer to phage in its normal state in the bacterial cell as immature or as prophage; it is suggested that these terms should not be used but instead the term non-infective be used.

Lwoff *et al.* (1950), Lwoff & Siminovitch (1951) found that the induction of phage lysis by means of reducing agents was dependent on the type of culture media used and they classified media accordingly as 'active' and 'inactive'. They found that broth was inactive for the strain of *B. megaterium* with which they were working. Lwoff (1952) discovered that the ability to bring about phage induction was dependent on the cationic balance of the medium and found that copper favoured induction by reducing agents and that cobalt was antagonistic to it. He showed that the concentration of thiolacetate necessary to induce lysis was indirectly related to the amount of copper present and directly to the amount of cobalt present. A different position existed in the salmonella cultures I examined. Broth was quite a satisfactory medium to demonstrate inducing effects, copper and cobalt played no part in thiolacetate induction, and of the reducing agents tested only those containing SH groups, sodium thiolacetate and glutathione, were able to induce phage lysis. The method of induction by thiolacetate and glutathione cannot be explained simply by alteration in the eH of the medium since it could not be obtained by all reducing agents. However, it was dependent on the amount of thiolacetate present and on the eH of the medium in some way since the inducing effects were not obtained in anaerobic cultures, although anaerobiosis had no effect on the liberation of free phage by cultures growing in ordinary broth. It was noted, too, that the higher concentrations of thiolacetate often caused some induction of lysis in the earlier stages of the growth cycle, followed by inhibition of phage production later; this inhibition was also noted with ascorbic acid.

Substances such as proflavine, cobalt sulphate, and yeast extracts were examined in the hope that they might throw light on the part played by nucleic acids in phage production, but the results were negative. Sulphathiazole was the only chemotherapeutic agent studied that had any effect on the liberation of phage particles and, since the increase did not occur when *p*-aminobenzoic acid was added, or when a large bacterial inoculum was used, it seemed that the increased liberation of phage might be associated with the bacteriostatic action of the sulphonamides, i.e. by interfering with *p*-aminobenzoic acid metabolism. Since many phenomena are common to both bacterial and animal virus systems it is tempting to compare the effect of sulphathiazole on lysogenic bacteria with the clinical observation that the

administration of sulphonamides in some animal virus infections, e.g. canine distemper, may result in an exacerbation of clinical symptoms. It is probable, however, that this is taking the comparison a little too far.

Nitrogen mustard and mustard gas induced lysis in lysogenic cultures in a similar manner to ultraviolet and X-rays; this is another reason for considering that the general biological activity of the mustards closely resembles that of ultraviolet and X-rays. It is noteworthy that of all the substances tested nitrogen mustard and mustard gas were the only two that were lethal to free phage particles in concentrations that were not lethal to bacteria. They were, however, able to stimulate the formation of very large amounts of free phage if they were removed from the medium after they had been in contact with the bacteria for a period of time or to a lesser extent when concentrations which were not lethal to free phage were added to the medium. The induction of phage lysis by means of nitrogen mustard, too, appears not to be restricted to the salmonellas since Jacob (personal communication) has independently and simultaneously discovered that it also induces the same effect in *Pseudomonas pyocyanea*.

I am indebted to Miss J. M. Gavins for her excellent technical help and to Mr L. D. S. Williams for a supply of mustard gas.

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(Received 12 July 1952)

KLECZKOWSKI, J. & KLECZKOWSKI, A. (1953). *J. gen. Microbiol.* 8, 135-144.

The Behaviour of *Rhizobium* Bacteriophages during and after Exposure to Ultraviolet Radiation

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SUMMARY: After inactivation by ultraviolet radiation, particles of two *Rhizobium* bacteriophages interfered temporarily with the multiplication of active particles of the homologous phage, in liquid cultures of their respective host bacteria. Inactivated particles did not affect the number of plaques produced by active particles in bacterial cultures on agar.

No evidence was found that particles that were inactive singly became active when two or more of them infected the same bacterial cell.

The rate of inactivation approximated closely to that of a first-order reaction. Exposing infected bacteria to visible light increased the residual activities of irradiated phage preparations by amounts equivalent to decreasing the doses of ultraviolet irradiation by a constant factor. Exposing either the irradiated phage preparations or the bacterial cultures separately to visible light had no effect.

Those ultraviolet irradiated phage particles which remained active were so altered that they became relatively unstable.

Much work has been done on the effects of ultraviolet radiation on bacteriophages of *Bacterium coli*, but relatively little on others. The course of inactivation, i.e. of becoming unable to multiply and cause lysis, was found to approximate to that of a first-order reaction, though slight deviations have been recorded (Latarjet & Wahl, 1945; Latarjet & Morenne, 1951). Some inactivated phages have proved able to interfere with multiplication of host bacteria and of active phages (Luria & Delbrück, 1942). Residual activity of some irradiated phage preparations could be increased by increasing their concentration at which they were brought into contact with susceptible bacteria ('multiplicity reactivation' Luria & Dulbecco, 1949) or by exposure of infected host bacteria to visible light ('photo-reactivation' Dulbecco, 1950).

The work described in the present paper was started to see how far these phenomena applied to bacteriophages of strains of *Rhizobium* and was done simultaneously with a comparable work on plant viruses (Bawden & Kleczkowski, 1953). During the work a new phenomenon was encountered; when irradiated phage preparations were stored their residual activity decreased at a much higher rate than did the activity of unirradiated phage preparation stored in the same conditions. A few experiments were made to study some details of this phenomenon.

MATERIAL AND METHODS

Two strains, 817 and Cl₅, of pea and clover nodule bacteria, respectively, and their two homologous bacteriophages, 817 and Cl₅, were used. Each bacteria strain was lysed by its homologous phage but not by the other. The bacterial strains, the bacteriophages, media and the 'poured plate' method of

producing plaques, have been described previously (Kleczkowska, 1945; Kleczkowski & Kleczkowski, 1952). Phage cultures were lysed liquid bacterial cultures passed through a Chamberland L3 filter and stored at 2°.

The source of ultraviolet (u.v.) radiation was a low-pressure mercury discharge lamp made by the Thermal Syndicate Ltd. and fitted with a cylindrical chromium-plated reflector. According to the makers' specifications the intensity of radiation, 99 % of which was of wave-length 2536 Å, was 121 μ W./sq.cm. at a distance of 100 cm.

Undiluted phage cultures were irradiated as layers 0.15 cm. deep in a Petri dish. They were exposed to the lamp at a distance of 20 cm. and rocked during the whole time of exposure. The rocking is assumed to have ensured that all phage particles were equally exposed to the radiation.

The intensity of radiation and its absorption by irradiated materials were not measured. The measurements would be useless because the amount of absorption by phage particles, as distinct from that caused by other constituents of crude bacterial lysates, would still remain unknown.

RESULTS

Interference by inactivated phage with multiplication of active phage and of host bacteria

This problem is considered first because if there were any interference it might need to be allowed for whenever residual activity of an irradiated phage preparation is tested.

The presence of inactivated phage does not interfere with formation of plaques by active phage. This is shown by the fact that adding untreated phage to an irradiated phage preparation, which is then incubated suitably diluted in a culture of host bacteria and plated, has no effect on the number of plaques. Nor is the plaque number affected by adding an irradiated phage preparation to an equal volume of a culture of host bacteria in which untreated phage preparation is then diluted and plated. Thus no active phage particles are destroyed or otherwise made permanently unable to multiply. On the other hand, irradiated phage preparations can stop completely, though temporarily, the multiplication of active phage in liquid cultures of host bacteria and can make the host bacteria unable to multiply and form colonies on agar medium.

Table 1 shows that an irradiated preparation of phage 317 prevented the multiplication of active phage 317 for at least 3 hr., during which the phage concentration of the control increased 30 times. However, this was so only when the irradiated phage preparation was added undiluted to an equal volume of a 24 hr. bacterial culture, i.e. when the number of inactive phage particles in the final mixture was more than 35 times that of the number of bacteria and 500,000 times that of the number of active phage particles. When the concentration of the irradiated phage preparation was decreased to one-quarter, phage multiplication was not completely inhibited and there was no inhibition when the concentration was decreased to one-sixteenth.

After 24 hr. incubation, the concentration of active phage in the culture containing even the most concentrated irradiated phage was almost as high as that of the control. It is obvious, therefore, that the inhibitory effect of the irradiated phage preparation lasted less than 24 hr.

Table 1. *Effect of an ultraviolet-irradiated preparation of phage 317 on multiplication of phage 317*

Materials. Untreated preparation of 317 phage: 5×10^8 plaques/ml. U.v. irradiated preparation of 317 phage: irradiation time was 20 min.; activity fell from 5×10^8 to 50 plaques/ml. U.v. irradiated preparation of Cl_5 phage: irradiation time was 20 min.; activity fell from 18×10^8 to 80 plaques/ml. 24 hr. culture of 317 bacteria: 15×10^8 cells/ml. (haemocytometer count). U.v. irradiated medium: irradiation time was 20 min.

The mixtures: 24 hr. culture of 317 bacteria + an equal volume of:	Phage concentration in the mixtures (in terms of numbers of plaques/ml.)		
	Immediately	After 3 hr.	After 24 hr.
Irradiated 317 phage (undiluted)	530	460	39×10^8 *
Irradiated 317 phage (dil. 1/4†)	600	1,500	—
Irradiated 317 phage (dil. 1/16†)	550	19,000	—
Irradiated Cl_5 (undiluted)	560	7,500	52×10^8
Untreated Cl_5 (undiluted)	500	15,000	54×10^8
Irradiated medium	570	8,000	50×10^8
Untreated medium	550	16,500	53×10^8

One volume of the untreated preparation of 317 phage diluted 1/10⁵ was added to nine volumes of each mixture. Samples of the mixtures were taken for assay of concentration of phage 317 by the plaque count method immediately, after 3 hr. and after 24 hr. incubation at 25°.

* The mean diameter of the plaques was about half that of the control.

† Diluted in untreated medium.

Although after 24 hr. incubation the culture containing irradiated phage produced almost as many plaques as did the control phage culture, the diameter of the plaques (mean = 1.9 mm.) was smaller than that of the control (mean = 3.4 mm.). No explanation of this phenomenon can be offered. It is not caused by a phage mutation, because phage isolated from the small plaques produced plaques of normal size.

Table 2 shows that u.v. inactivated phage Cl_5 also temporarily prevented the multiplication of active phage Cl_5 in a liquid culture of Cl_5 bacteria. Comparison of Tables 1 and 2 shows that the multiplication of active phage was prevented only by irradiated preparations of the homologous phage. Irradiated preparations of the other inhibited only to the same extent as did the irradiated medium.

This apparent specificity probably occurs because each of the two bacterial strains was susceptible to only one of the two phages used. Luria & Delbrück (1942), who used one bacterial host susceptible to two different phages, found that irradiated preparations of one phage could slow the multiplication of either phage in liquid cultures of their common host, whereas irradiated preparations of the other had no effect on multiplication of either. It appears, therefore, that if a u.v. irradiated phage can interfere with the multiplication

of active phage, it does so only in a bacterial strain which is its host and then it interferes with the multiplication of any phage in it.

Luria & Delbrück (1942) found that the u.v. inactivated phage that interfered with phage multiplication also rendered the bacteria unable to multiply, whereas the other inactivated phage had no such effect. Moreover, the ability of the irradiated phage to interfere with phage multiplication could be destroyed by excess of u.v. irradiation, when their ability to make their host

Table 2. *Effect of an ultraviolet irradiated preparation of Cl₈ phage on multiplication of Cl₈ phage*

Materials. Untreated preparation of Cl₈ phage: 18×10^8 plaques/ml. U.v. irradiated preparation of Cl₈ phage: irradiation time was 20 min.; activity fell from 18×10^8 to 80 plaques/ml. U.v. irradiated preparation of 817 phage: irradiation time was 20 min.; activity fell from 5×10^8 to 50 plaques/ml. 24 hr. culture of Cl₈ bacteria: 16×10^6 cells/ml. (haemocytometer count). U.v. irradiated medium: irradiation time was 20 min.

The mixtures: 24 hr. culture of Cl ₈ bacteria + an equal volume of:	Phage concentration in the mixtures (in terms of numbers of plaques/ml.)	
	Immediately	After 5 hr.
Irradiated phage Cl ₈	950	810
Irradiated phage 817	890	2500
Untreated phage 817	930	6700
Irradiated medium	860	3000
Untreated medium	870	7000

One volume of the untreated preparation of Cl₈ phage diluted $1/10^5$ was added to nine volumes of each mixture. Samples of the mixtures were taken for assay of concentration of phage Cl₈ by the plaque count method immediately and after 5 hr. incubation at 25°.

bacteria unable to multiply also disappeared. It is probable, therefore, that the interference with both phage and host multiplication are expressions of the same disturbance of host metabolism caused by the u.v. inactivated phage. If so, u.v. inactivated *Rhizobium* phages would be expected to be able to interfere with multiplication of their host bacteria, and Table 3 shows that addition of an irradiated preparation of phage 817 to an equal volume of a 24 hr. culture of 817 bacteria did decrease considerably the number of colonies formed by the bacteria on agar medium. The decrease was probably caused by u.v. inactivated phage and not by the remaining active phage or by some other constituent of the irradiated phage preparation. The number of plaques formed by the irradiated preparation was 50/ml. Thus, if the proportion of active phage particles that formed plaques when the preparation was assayed was $1/m$, the number of active phage particles per ml. of the mixture with the bacterial culture was $25m$. The results of an electron microscopic study by Luria, Williams & Backus (1951) suggest that the value of m is not greater than 10, so that the number of active phage particles per ml. of the mixture with bacterial culture was probably not greater than 250, whereas the approximate number of bacterial cells per ml. of the mixture rendered unable to form colonies was 1×10^6 . Therefore, some constituent of the irradiated preparation other than active phage must have made them unable

to do so. The fact that irradiated medium or irradiated (as well as unirradiated) preparations of a heterologous phage (Cl_5) caused no drop in the number of colonies, shows that the constituent of the irradiated preparation of the homologous phage (317) that caused the drop was probably inactivated phage 317.

Table 3. *Effect of an ultraviolet irradiated preparation of phage 317 on multiplication of its host bacteria*

Materials. All the materials used in this experiment were the same as those used in the experiments shown in Tables 1 and 2.

The mixtures: 24 hr. culture of 317 bacteria + an equal volume of:	Mean numbers of colonies/plate formed by the mixtures used at a dilution of	
	1/10 ⁴	1/10 ⁵
Untreated phage 317	42	4.5
Irradiated phage 317	264	25
Untreated phage Cl_5	380	42
Irradiated phage Cl_5	370	39
Untreated medium	355	36
Irradiated medium	363	39

The mixtures were incubated for 15 min. at room temperature then diluted 1/10⁴ and 1/10⁵ in untreated medium, and 1 ml. of each dilution was plated by mixing with 9 ml. of melted agar medium cooled to 42° and pouring into a Petri dish which was then incubated for 7 days at 25°. There were four replications of each treatment.

However, irradiated medium or irradiated preparations of the heterologous phage can slow bacterial multiplication in liquid cultures if mixed in equal volumes. This agrees with other observations that u.v. irradiation makes media less suitable for bacterial growth, an effect usually attributed to the action of peroxides formed by u.v. irradiation (Coblentz & Fulton, 1924; Bedford, 1927; Proks, 1933; Wyss, Haas, Clark & Stone, 1950). The slight inhibitory effect on phage multiplication given by irradiated medium and irradiated preparations of heterologous phages (Tables 1 and 2) probably resulted from their effect on growth of the host bacteria.

The common feature of the effect of u.v. inactivated phages, on the one hand, and of such inhibitors as ribonuclease or some polysaccharides (Kleczkowski & Kleczkowski, 1952), on the other, is that they interfere with phage multiplication in liquid cultures of host bacteria while having no effect on the numbers of plaques formed on agar. This effect on the former and not the latter, showing an apparent contradiction, has been discussed elsewhere (Kleczkowski & Kleczkowski, 1952).

Tests for 'multiplicity reactivation'

The 'multiplicity reactivation' observed with a few coli bacteriophages (Luria & Dulbecco, 1949) was not observed with the *Rhizobium* bacteriophages used in this work. The numbers of plaques produced by u.v. irradiated preparations of these phages did not increase with the increasing concentration at which they were brought into contact with host bacteria before plating. The phenomenon of 'multiplicity reactivation' does not seem to be very common. It

was observed only in a proportion of a few coli phages tested by Luria & Dulbecco (1949), and it could not be observed with a *Staphylococcus* phage (Price, 1950), or with three different plant viruses (Bawden & Kleczkowski, 1953).

The rate of inactivation and the effect of visible light

Table 4 shows that the rate of inactivation of the 317 phage by u.v. radiation approximates closely to that of a first-order reaction because the value of k , obtained from the equation $p = e^{-kt}$ (where p is the proportion of remaining activity and t the time of exposure), was almost constant when experimental conditions were constant.

Table 4. *The rate of inactivation of phage 317*

Time of exposure to u.v. radiation (min.)	Dilution at which the preparation was plated	Total nos. of plaques formed on 14 plates*	Proportion of remaining activity	$k†$
0	1/10 ⁸	519	1.00	—
0.283	1/10 ⁸	278	0.54	2.15
1	1/10 ⁷	498	0.096	2.34
2	1/10 ⁶	713	0.014	2.13
4	1/10 ⁴	956	0.00018	2.16

* There were seven separate experiments with the same times of exposure. In each experiment two platings were made with each preparation giving 14 as the total number of platings.

† The value of k is obtained from the equation $p = e^{-kt}$, where p is the proportion of remaining activity and t is the time of exposure (in minutes).

Table 5. *The effect of visible light on the activity of ultraviolet irradiated 317 phage*

Time of irradiation of phage (min.)	Dilution at which the preparation was plated	Plates not exposed to visible light			Plates exposed for 8 hr. to daylight immediately after plating		
		Total nos. of plaques formed on 4 plates	Proportion of remaining activity	k^*	Total nos. of plaques formed on 4 plates	Proportion of remaining activity	k^*
0	1/10 ⁷	165	1.00	—	158	1.00	—
2.25	1/10 ⁶	131	0.0079	2.15	336	0.021	1.72
4.5	1/10 ⁵	130	0.000079	2.10	765	0.00048	1.70

* Obtained as in Table 4.

Table 5 shows that a smaller value of k is obtained if the agar plates inoculated with mixtures of host bacteria and irradiated phage are exposed for some time to visible light than if they are kept all the time in darkness. In each condition, however, the value of k is constant. The decrease in the value of k arises from the fact that u.v. irradiated phage shows increased residual activity if the infected host bacteria are exposed to visible light. The phenomenon is shown by *Rhizobium* phage in all details as observed by Dulbecco (1950) with a number of coli phages and described as 'photo-reactivation'. The constancy of k under constant conditions of exposure to visible light or in its absence agrees with the so-called 'dose reduction prin-

ciple' of Kelner (1949), according to which exposure to visible light is equivalent to reducing the dose of u.v. radiation by a constant factor. The principle was formulated for u.v. irradiated bacteria and found by Dulbecco (1950) to apply to u.v. irradiated coli phages and by Bawden & Kleczkowski (1953) to some u.v. irradiated plant viruses.

Visible light increases the residual activity of u.v. irradiated phage preparations only if the infected host bacteria are exposed to it. Exposure of the irradiated phage preparations, or of the host bacteria, or of both, separately, i.e. before they are mixed, has no effect. Similarly, the residual activity of u.v. irradiated preparations of some plant viruses is increased by exposing the inoculated host plant to visible light, whereas exposure of irradiated virus preparations *in vitro*, or of the host plant before inoculation, has no such effect (Bawden & Kleczkowski, 1953).

Visible light can counteract lethal and some other effects of u.v. radiation on unicellular (see Kelner, 1951) and on some multicellular (Bawden & Kleczkowski, 1952) organisms by acting on them directly, whereas it can counteract comparable effects of u.v. radiation on bacteriophages and plant viruses only when they have combined with their cellular host organisms. Cellular structure is thus apparently essential for the occurrence of this phenomenon.

The fact that illumination of infected host bacteria cannot increase residual activity of u.v. irradiated phage above a certain limit, so that a proportion of phage particles will remain inactive irrespective of the amount of illumination, led Dulbecco (1950) to conclude that there are two different kinds of injury inflicted by u.v. radiation on phage particles: 'photoreactivable' and 'nonphotoreactivable'. The conclusion that there are at least two and possibly more different kinds of injury indeed seems inescapable. The fact that the value of k is constant when the conditions of illumination are constant means that the process of inflicting each kind of injury is a separate first-order reaction. Conclusions that can be drawn from this are given elsewhere (Bawden & Kleczkowski, 1953).

Relative instability of active phage in irradiated preparations

Tables 6 and 7 show that when u.v. irradiated and unirradiated phage preparations are incubated in identical conditions, the former lose their activity at a proportionally higher rate than the latter. The more a preparation is irradiated the higher is the relative rate of the decrease of its remaining activity (Table 6). The difference between irradiated and control preparations is noticeable when the incubation temperature is near 0°, but it becomes more obvious as the temperature increases, i.e. when both irradiated and control preparations become less stable (Table 7).

It is concluded that irradiated phage particles that still remain active are less stable than those in control preparations. Their relative instability does not result from the presence in the medium of any materials that are produced by u.v. radiation and are harmful to phage particles, because unirradiated phage preparations diluted in irradiated preparations are as stable as their

controls. The instability is, therefore, a property of the irradiated phage particle and it is a result of either a change in the structure of the particle caused by u.v. radiation, or a selective effect of the radiation. The latter possibility seems unlikely, because it necessitates the assumption that the

Table 6. *Effect of incubation at 34° of preparations of phage 317 ultraviolet irradiated for various lengths of time*

Time of irradiation (min.)	Dilution at which the preparation was plated	Numbers of plaques formed on 4 plates by samples plated		Drop in activity to
		Immediately after irradiation	After 3 hr. incubation at 34° following irradiation	
0	1/10 ⁷	305	295	97 %
1.75	1/10 ⁶	84	60	71 %
3.5	1/10 ⁴	225	74	38 %
5.0	1/10 ³	980	257	26 %

Table 7. *Effect of incubating an ultraviolet irradiated preparation of 317 phage at different temperatures*

	Untreated phage		Phage irradiated for 4 min.	
	Nos. of plaques on 4 plates	Remaining proportion of original activity (%)	Nos. of plaques on 4 plates	Remaining proportion of original activity (%)
A sample plated				
Immediately	350	100	660	100
After 24 hr. incubation at:				
2°	385	110	520	80
20°	370	105	390	60
34°	290	80	45	7

Samples of unirradiated phage preparation were plated at a dilution of 1/10⁷ and those of the irradiated preparation at 1/10³.

more stable particles are more easily inactivated by u.v. radiation than the less stable ones. Moreover, it would have to be assumed that phage particles differ in susceptibility to u.v. radiation, and this means that the value of *k* should decrease as the residual activity of irradiated phage preparations decreases to the values reached in the experiments shown in Tables 6 and 7, whereas in fact the value of *k* remained approximately constant (Tables 4 and 5). It seems, therefore, more likely that u.v. radiation can so alter a phage particle that, although still active, it is rendered unstable.

The relative instability of particles that remain active after exposure to u.v. radiation is a phenomenon that does not seem to be limited to bacteriophages, for it has also been observed in preliminary experiments with trypsin (Kleczkowski, to be published). It was not observed with a few u.v. irradiated plant viruses that were tested (Bawden & Kleczkowski, 1953), but the failure

may have resulted from failure to adjust experimental conditions to the much greater stability of the viruses used.

Alper (1952) has just reported that inactivation of a phage by X-rays still goes on after the irradiation of its preparation has ended. Two different causes of this could be distinguished. In the first place X-rays alter phage particles making them susceptible to the inactivating effect of hydrogen peroxide, and, secondly, X-rays produce hydrogen peroxide in the medium. Diluting irradiated phage in unirradiated medium can, therefore, prevent its further quick inactivation.

Instability of residual activity of u.v. irradiated preparations of the *Rhizobium* bacteriophages is not likely to be caused by a similar combination of two causes, for diluting the preparations 1/1000 in unirradiated medium did not make them more stable. On the contrary, they even became less stable, suggesting the presence of some protective material in the crude bacterial lysate.

When residual activity of u.v. irradiated phage preparations falls as a result of ageing, the ability of the preparations to respond to exposure of infected host bacteria to visible light seems to remain unchanged. The ratio of the number of plaques formed with exposure to visible light to that formed without exposure does not seem to alter appreciably. It is concluded that irradiated phage particles that still are fully active and those active only if the infected host is exposed to visible light, both lose their activities at the same relative rate. The ability of an u.v. irradiated phage particle to be active only if the infected host is exposed to visible light may be a transition stage between fully active and fully inactive states, but there is no evidence for this.

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(Received 14 July 1952)

BAWDEN, F. C. & KLECZKOWSKI, A. (1953). *J. gen. Microbiol.* 8, 145-156.

The Behaviour of some Plant Viruses after Exposure to Ultraviolet Radiation

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SUMMARY: Preparations of tobacco mosaic virus (TMV) inactivated by ultraviolet radiation interfered slightly with infection by active tomato bushy stunt (BSV) and Rothamsted tobacco necrosis (RTNV) viruses, and much more so with active TMV. Similarly, inactivated RTNV interfered slightly with infection by TMV and more so with active RTNV. In contrast, inactivated BSV did not affect the numbers of lesions produced by active virus preparations.

The residual infectivity of irradiated preparations of RTNV and BSV was greater when inoculated plants were exposed to light than when they were kept in the dark. This occurs because of some light-sensitive mechanism in the host cells, and exposing the irradiated virus preparations to visible light did not affect their infectivity. Irradiated preparations of TMV had the same residual infectivity whether plants were placed in the light or dark after inoculation.

Although the three viruses have particles of different sizes and shapes, the course of inactivation by ultraviolet with each approximated closely to that of a first-order reaction.

Exposing preparations of some plant viruses to ultraviolet radiation destroys their infectivity without producing any gross changes in their physico-chemical or serological properties (Stanley, 1936; Bawden & Pirie, 1938*a, b*). Studies on the course of inactivation have led to the conclusion that it follows the course of a first-order reaction, which has sometimes been taken to indicate that inactivation is caused by a 'single hit'. The conclusion that infectivity decreases exponentially with time of irradiation has been reached whether workers have measured residual infectivity by assuming that numbers of local lesions are proportional to the concentration of infective virus (Price & Gowen, 1936; Lea & Smith, 1940), an assumption known to be invalid since Holmes (1929) first described the use of local lesions for quantitative work, or whether they have used some other method (Oster & McLaren, 1950). Despite the agreed conclusion, therefore, the problem cannot be taken as settled, particularly as deviations from an exponential function have not been tested to see whether they fall within experimental error. Because of this uncertainty, we have re-examined the course of inactivation, although this was not the main purpose of the experiments we describe. These were done primarily to see if phenomena described with irradiated preparations of some bacteriophages are shown by plant viruses. Experiments were made to test for three phenomena, namely: (1) whether inactivated virus interferes with the establishment of active virus, a phenomenon that has been demonstrated with several animal viruses (Henle, 1950) as well as with bacteriophages (Luria & Delbrück, 1942; Kleczkowski & Kleczkowski, 1953); (2) whether irradiated preparations are proportionally more infective when concentrated than when dilute, a

phenomenon described and called 'multiplicity reactivation' by Luria & Dulbecco (1949); (3) whether visible light affects the infectivity of irradiated preparations, a phenomenon described and called 'photoreactivation' by Dulbecco (1950).

MATERIALS AND METHODS

Purified preparations of three viruses were used; those of tobacco mosaic virus (TMV) and tomato bushy stunt virus (BSV) were made by precipitation methods and those of the Rothamsted culture of a tobacco necrosis virus (RTNV) by differential ultracentrifugation.

The test plants were those that react with countable necrotic local lesions, *Nicotiana glutinosa* with TMV and BSV, and French bean (*Phaseolus vulgaris* var. Prince) with RTNV. Inocula to be compared were rubbed as evenly as possible over the upper leaf surfaces with the forefinger. In most tests, eight inocula were compared; these were applied to half-leaves distributed among the test plants so that errors arising from differences in susceptibility between plants, or, with *Nicotiana glutinosa*, between leaves occupying different positions on the stem, could be eliminated (Kleczkowski, 1950). Each inoculum was rubbed over twelve half leaves. Sometimes twelve inocula were compared on *N. glutinosa*; then each was applied to nine half leaves, nine plants being used, each with six leaves. In some experiments to find the effect of exposing inoculated plants to visible light, inoculum of irradiated and control virus could be used at only one concentration. No exact quantitative conclusions were drawn from comparisons of lesion numbers obtained in such experiments, but by suitably adjusting the concentration of the two inocula qualitative effects were clearly obvious. Whenever results were analysed statistically, the numbers of lesions were transformed according to the formula $y = \log_{10}(x + 5)$, in which x is the number of local lesions produced per half leaf, and analyses of variances were done on the transformations (Kleczkowski, 1949).

Two sources of ultraviolet radiation were used. One, an 'Osira' 80 W. lamp made by the General Electric Company Ltd., with its glass envelope removed, gives a polychromatic radiation. The other, a low-pressure mercury-discharge lamp made by the Thermal Syndicate Ltd., fitted with a chromium-plated cylindrical reflector, gives a radiation 99 % of which is of wavelength 2536 Å. No qualitative differences were noticed between the behaviour of the two lamps in any of our experiments. Virus solutions were irradiated as layers 0.14 cm. deep in Petri dishes, at a distance of 20 cm. from the lamps. In these conditions the intensity of radiation with the low-pressure lamp was $870 \mu\text{W./sq.cm.}$ The dishes were rocked during the whole exposure to radiation and this is assumed to have ensured that all virus particles had equal opportunities to absorb the same amount of radiation.

EXPERIMENTAL

Interference between active and inactivated virus

The ability of virus inactivated by ultraviolet to interfere with infection by active virus was tested by comparing the relative infectivities of solutions of control virus diluted in water with those similarly diluted in solutions of

inactivated virus at various concentrations. Some typical results are recorded in Table 1, which shows the sums of actual numbers of local lesions produced and the sums of values transformed for statistical analyses. As found with different bacteriophages (Luria & Delbrück, 1942), inactivated preparations of different plant viruses also differ in their behaviour. Inactivated BSV had no effect on the numbers of lesions produced by active preparations of either BSV or TMV, whereas inactivated preparations of TMV and RTNV decreased the numbers produced by all the active viruses with which they were mixed. However, inactivated TMV interfered much more with active TMV, and inactivated RTNV with active RTNV, than either did with other viruses.

Table 1. *Interference of inactive with active viruses*

Exp. no.	Contents of inocula (mg./l.)		Total numbers of lesions on twelve half-leaves	The sums of twelve transformed numbers of lesions†	Host plants
	Untreated virus	Inactivated virus*			
1	2 TMV	5000 TMV	270	17.11	Nicotiana glutinosa
	2 TMV	5000 BSV	630	20.34	
	2 TMV	—	632	20.52	
2	0.2 TMV	5000 TMV	30	—	
	0.2 TMV	5000 BSV	104	—	
	0.2 TMV	—	119	—	
3	5 BSV	5000 TMV	195	15.30	
	5 BSV	5000 BSV	359	17.17	
	5 BSV	—	371	17.51	
4	0.5 BSV	5000 TMV	38	—	French bean
	0.5 BSV	5000 BSV	48	—	
	0.5 BSV	—	48	—	
5	1 TMV	4000 TMV	207	15.58	
	1 TMV	4000 RTNV	401	18.21	
	1 TMV	—	468	19.05	
6	0.2 TMV	4000 TMV	52	11.19	
	0.2 TMV	4000 RTNV	139	13.94	
	0.2 TMV	—	196	15.06	
7	1 RTNV	4000 TMV	440	19.12	French bean
	1 RTNV	4000 RTNV	272	17.19	
	1 RTNV	—	580	20.47	
8	0.2 RTNV	4000 TMV	107	—	
	0.2 RTNV	4000 RTNV	44	—	
	0.2 RTNV	—	193	—	

* The viruses were irradiated at 1% solutions. The times of irradiation were: TMV, 3 hr., BSV, $\frac{1}{2}$ hr., and RTNV, 1 hr.

† The transformation was $y = \log_{10}(x+5)$, where x 's are the numbers of lesions per half-leaf.

Statistically significant differences between the sums of transformed numbers for different levels of probability (P) obtained from a table of 'Student's' distribution of t .

Exp. no.	P			
	0.05	0.02	0.01	0.001
1	1.76	2.12	2.38	3.16
3	1.88	2.27	2.55	3.43
5	2.25	2.70	3.03	4.01
6	1.77	2.13	2.38	3.16
7	1.29	1.56	1.74	2.31

The interpretation of these results is uncertain. Many substances interfere with infection by plant viruses, and several are known that are powerful inhibitors of TMV when used at much smaller concentrations than 0.5 mg./ml., the smallest at which inactivated TMV had any demonstrable effect. These substances do not act specifically against individual viruses, but the extent of their inhibitory power does vary with the identity of the host plant. The mechanism of inhibition is undetermined, but there is growing evidence that the inhibitors act by altering the host-cell metabolism rather than by directly affecting the virus particles (Gupta & Price 1950, 1952; Bawden & Freeman 1952). It is reasonable to assume that the inhibiting effect of inactivated TMV on BSV and RTNV is analogous to that of these other substances. There is, however, evidence of an additional and larger effect operating specifically between inactivated and active TMV and between inactivated and active RTNV. At first sight this suggests that two different mechanisms may be involved, but this is not necessarily so. Both the specific and unspecific interference could result from the same cause, and our results are most simply explained by postulating that inactivated TMV and RTNV, but not inactivated BSV, affected the metabolism of leaf cells into which they were introduced, so that the cells became less favourable for any virus to become established but particularly so for one resembling the initial stimulus. We have no evidence that the inactivated plant viruses do affect cellular metabolism, but, by analogy with results from tests with irradiated bacteriophages, it is reasonable to assume they do. That bacteria can be changed by absorbing inactivated particles of some bacteriophages is shown by their subsequent inability to divide (Luria & Delbrück, 1942; Kleczkowski & Kleczkowski, 1953).

Interpreting similarities and differences between results with bacteriophages and with plant viruses is complicated because the initiation of infection with the two calls for such different conditions. To gain infection with plant viruses, host cells need to be wounded and other materials than virus particles can presumably also enter through the wounds. In contrast, bacteriophages infect uninjured bacteria and only bacteriophages and other materials that are specifically absorbed by bacteria can enter. This difference may explain the fact that a small non-specific inhibition is produced by irradiated TMV and RTNV, whereas no such effect has been observed with bacteriophages, with which interference by inactivated particles depends on the susceptibility of the bacteria to the particular phage (Kleczkowski & Kleczkowski, 1953). Another difference between the two is that, if inactivated particles interfere with infection by active particles of the same phage, they also seem to interfere equally with infection by other phages to which the bacterium is susceptible (Luria & Delbrück, 1942), whereas in *N. glutinosa*, which is a host plant for both TMV and RTNV, inactivated RTNV has much less effect on TMV than inactivated TMV. This difference is possibly explained by the fact that susceptible bacteria are more affected by the entry of inactivated virus particles, as is suggested by the inability of the cells to multiply, than are leaf cells of higher plants.

Inactivated particles of coli phages lose their ability to interfere with infec-

tion by active particles if they are exposed to too much ultraviolet (Luria & Delbrück, 1942). We have made no special tests for this phenomenon with plant viruses, but within the range of minutes to hours that we have irradiated preparations of TMV and RTNV inactivated particles seem equally effective. Interference occurs with preparations irradiated well beyond the point at which any residual infectivity is demonstrable and in those irradiated so that they retain about 1% of the original infectivity. It is the only phenomenon suggesting any interaction between individual virus particles that we have noted, and we found nothing to indicate that particles inactive singly could together become active. That such a thing might happen was suggested by Luria & Dulbecco (1949) to explain their results when irradiated preparations of some coli phages were mixed in various proportions with bacteria before being diluted and plated. They found that the numbers of plaques produced by irradiated preparations increased with increase in the ratio of the preparation to bacterial cells in the mixture before dilution.

The procedure used with bacteriophage is obviously inapplicable to plant viruses, but if multiple infections with particles inactive singly lead to virus multiplication, this should be detectable by comparing the dilution curves given by partially inactivated preparations with those given by unirradiated preparations. As the concentration of virus in the inoculum increases, the mean number of particles entering an infection site will be expected to increase, an expectation that is supported by the ability of concentrated preparations of inactivated TMV to interfere with the multiplication of active particles. By plotting the numbers of local lesions against logarithms of virus concentration, therefore, the phenomenon of multiplicity reactivation should show by the irradiated preparations giving a steeper curve than control preparations.

Tests with TMV, BSV and RTNV, irradiated for various times to give residual activities between 0.1 and 10% of the original preparations, gave no evidence that particles inactive singly could cause infection when acting jointly. All the irradiated preparations gave dilution curves of the same form as those given by control virus preparations, until the concentration of virus in the irradiated preparations reached about 0.1%. Then, instead of getting the steeper curve to be expected with multiplicity reactivation, the irradiated preparations gave flatter curves than the control preparations, because at this level the inactivated particles began to prevent infection with the active ones.

To account for the proportionally greater infectivity of concentrated preparations of their irradiated bacteriophages, Luria & Dulbecco (1949) suggested that particles contain several activities or 'genes' each of which can be destroyed singly, and that activities in different particles can replace one another, so that two particles in which different activities are destroyed still supply the full complement needed in a bacterium for phage production. Were this interpretation correct, it seems likely that structures of this type would be typical rather than restricted to a few viruses. The phenomenon of proportionally greater infectivity at high concentrations, however, seems to occur rarely, for it was not found with all coli phages (Luria & Dulbecco, 1949), with a staphylococcal phage (Price, 1950), with two phages of *Rhizobium* sp.

(Kleczkowski & Kleczkowski, 1953), or with the plant viruses we have studied. When irradiated preparations of bacteriophages at different dilutions are mixed with bacteria, it is not only the ratio of phage particles to bacterial cells that alters, but also the ratio of everything else in the preparation. Ultraviolet may change other components than virus particles, and infection with the coli phages studied by Luria & Dulbecco may be facilitated when these changed components exceed a critical concentration.

Effects of visible light on infectivity

Before making detailed measurements on rates of inactivation, tests were done to see whether such measurements would be affected by the treatments to which the irradiated virus preparations and inoculated plants were subjected. Kelner (1949, 1951) has shown that the proportion of irradiated fungal spores and bacteria that multiply is greater if cultures are exposed to visible light than if they are kept in darkness, and Dulbecco (1950) that the residual activity of irradiated bacteriophage is greater if the inoculated bacteria are illuminated than if kept dark. With two of the plant viruses, RTNV and BSV, we obtained results similar to those obtained with the bacteriophages. When irradiated preparations of these two viruses were exposed to visible light *in vitro*, their activity was unaffected, but when plants were exposed to light after inoculation the residual infectivity was increased. TMV behaved differently, and exposing either the irradiated virus preparations themselves or the inoculated plants to visible light did not increase the residual infectivity.

Various factors complicate experiments to test the effect of exposing inoculated plants to light on the infectivity of irradiated virus preparations. There is no direct proportionality between numbers of local lesions and concentration of infective virus, individual plants may produce widely different numbers of lesions when rubbed with the same inoculum, and the numbers produced by unirradiated inocula depend on the treatment of the plants, including their illumination. Conclusions can be drawn only from comparisons between irradiated and unirradiated inocula applied to similarly treated plants in experiments statistically designed.

Table 2 shows the results of one experiment with each of the three viruses, which were irradiated so that their residual infectivity was about 0.5% of the original when the host plants were kept in darkness after inoculation. Control virus preparations were used at a hundred times the dilution of the irradiated preparations, so that both produced numbers of lesions that did not differ greatly. Tests were made with plants, some of which had been in darkness for a day before they were inoculated and some of which had been maintained under normal glasshouse conditions. After inoculation each batch of plants was divided into two, and one lot was kept in darkness for a day whereas the other was kept under ordinary glasshouse conditions. The experiments with all three viruses confirm previous results (Samuel & Bald, 1933; Bawden & Roberts, 1948) that keeping plants in darkness before inoculation increases their susceptibility, and they show that the numbers of lesions produced by irradiated and unirradiated inocula are increased equally by this treatment.

A period in the dark after inoculation with unirradiated inocula had less effect, but, as also found previously (Bawden & Roberts, 1948), usually decreased the numbers of lesions. With TMV the decrease was the same with both irradiated and unirradiated inocula, so that the ratio between the numbers of lesions produced by the two inocula was similar whether the plants were illuminated or not. By contrast, the irradiated inocula of BSV and RTNV produced fewer lesions than unirradiated inocula when the inoculated plants were put in the dark, but more when the plants were put in the light.

Table 2. *Effects of exposing host plants to daylight before and/or after inoculation with untreated and with ultraviolet irradiated virus preparations*

		RTNV on French bean		BSV on <i>N. glutinosa</i>		TMV on <i>N. glutinosa</i>	
		Before inoculation		Before inoculation		Before inoculation	
		Dark	Light	Dark	Light	Dark	Light
Dark after inoculation	A	24	6.5	19.5	3	23	13
	B	14.5	2.5	8	0.7	10	8
Light after inoculation	A	24	11.5	84	7.5	50	12.5
	B	41	26	85	9	34	7

The numbers are mean numbers of lesions per leaf obtained on twelve to fourteen half-leaves.

A, untreated virus at 1 mg./l. RTNV, 5 mg./l. BSV, 1 mg./l. TMV.

B, irradiated virus at 100 mg./l. RTNV, 500 mg./l. BSV, 100 mg./l. TMV.

Irradiation: RTNV 0.01 % 1 min. 20 sec.

BSV 0.1 % 1 min. 30 sec.

TMV 0.1 % 7 min.

Light: the plants were exposed to uncontrolled daylight.

Dark: the plants were kept in darkness for 24 hr.

The numbers of lesions produced by irradiated preparations of BSV and RTNV were increased only when plants were exposed to light soon after they were inoculated. The fate of a virus particle, that is, whether it will become established and multiply, is determined within a few hours; particles seem unable to remain dormant in cells if conditions are temporarily unfavourable and then become established 24 hr. later when conditions are made more favourable by exposing the leaves to light. We have made no detailed experiments on the amount of light needed to give the maximum infectivity with irradiated preparations, but most of the increase occurs when plants are exposed for 3 hr. to daylight under glass, when the intensity at leaf level is around 800 f.c. Although to get a response inoculated plants must be exposed to light without delay, the irradiated inocula need not be used immediately. Tests with BSV and RTNV 14 days after irradiation gave the same relative differences between plants kept in the light and dark after inoculation as with tests made within an hour of irradiation.

The conditions in which infection with irradiated BSV and RTNV is favoured are those in which irradiated leaves can also counteract damage to themselves. Bean leaves exposed to ultraviolet remain apparently unharmed if later kept in the light, whereas their epidermal cells die if they are kept in the dark

(Bawden & Kleczkowski, 1952). The mechanism responsible for increasing the infectivity of the irradiated virus preparations is almost certainly the same as that which counteracts the lethal effects of radiation on the cells. Whether it operates by reversing changes in the virus particles caused by ultraviolet, or by making conditions such that damaged virus particles can still function, is unknown. It seems that the host-cell system responsible is one that operates only in light and does not lead to any accumulation of products that themselves counteract the damaging effects of ultraviolet. This is suggested by the fact that plants kept in the light before inoculation produced proportionally as many more lesions when exposed to visible light after inoculation with irradiated viruses as did plants kept in the dark until they were inoculated. The effect of excluding light before inoculation in increasing susceptibility seems a different phenomenon and may occur simply because the removal of photosynthetic products facilitates the entry of particles when leaves are rubbed.

Rates of inactivation

Experiments in which plants inoculated with virus preparations irradiated for various times were separated into two lots, one of which was kept in the light and the other in the dark, showed that the post-inoculation treatment, provided it was kept constant, was unimportant in studying the course of inactivation. With TMV the rate of inactivation was the same whether plants were kept in the light or dark, whereas with RTNV and BSV it was greater by about 20 % when plants were in the dark than when in the light. The effect was constant throughout the course of irradiation and, with these two viruses, exposing the inoculated plants to visible light was quantitatively equivalent to decreasing the dose of ultraviolet by a constant factor. Kelner (1949) described this phenomenon with irradiated bacteria as the 'dose reduction principle', and it also applies to bacteriophages (Dulbecco, 1950; Kleczkowski & Kleczkowski, 1953).

Table 3 shows the results of experiments in which the residual activity of preparations was measured after various times of irradiation and inoculated plants were kept under normal glasshouse conditions. The course of inactivation closely follows that of a first-order reaction, for variations in the values of k , obtained from the equation $\log_{10} r = -0.4343kt$, in which r is the residual activity as a proportion of the original and t is the time of irradiation in minutes, in any experiment are small and could fall within chance variations from a constant value. The values of $\log_{10} r$ given in Table 3 were obtained by graphic interpolation from dilution curves and this method does not allow definite conclusions as to whether deviations fall within experimental error. A statistical method will be described elsewhere (Kleczkowski, 1953) for testing the compatibility of lesion counts with different hypotheses, and this has been applied to Exps. 1, 2 and 4 from Table 3. The test showed that the variations in k in Exps. 1 and 4 do not depart significantly from a constant value; they do in Exp. 2, but the deviation is only slight. Thus it can be assumed that the course of inactivation does approximate closely to that of a first-order reaction, which has also been demonstrated with bacteriophages

Table 3. *The course of inactivation of the viruses by ultraviolet radiation*

Ultraviolet irradiation of virus preparations		Virus content of inoculum (Log ₁₀ of per- centage of concentration)	Nos. of lesions per half-leaf	log ₁₀ <i>r</i> [*]	<i>k</i> [†]
Concentra- tion of the preparation (%)	Time of irradiation (min.)				
Exp. 1. RTNV on French bean					
1.0	2.48	-3.0	134	-0.54	0.50
	4.24		100	-0.85	0.46
	6.00		65	-1.12	0.43
	7.77		29	-1.65	0.49
	Unirradiated	-3.7	114		
	control	-4.2	59		
		-4.7	27		
		-5.2	8		
Exp. 2. RTNV on French bean					
0.1	0.58	-3.0	95	-0.89	3.5
	1.00		39.5	-1.41	3.3
	1.42		17.5	-1.75	2.8
	1.83		4.5	-2.26	2.8
	Unirradiated	-3.7	125		
	control	-4.2	56.5		
		-4.7	19.5		
		-5.2	5		
Exp. 3. RTNV on French bean					
0.01	0.50	-3.0	70	-1.00	4.6
	0.87		19	-1.83	4.8
	1.22		3	-2.60	4.9
	1.58		1.7	-3.20	4.7
	Unirradiated	-3.7	104		
	control	-4.2	47		
		-4.7	24		
		-5.2	7		
Exp. 4. TMV on <i>N. glutinosa</i>					
0.5	3.85	-2.5	122	-0.62	0.37
	7.70		100	-0.78	0.23
	11.55		53	-1.27	0.25
	15.40		26	-1.80	0.27
0.01	0.575	-2.5	123	-0.62	2.5
	1.150		64	-1.03	2.1
	1.725		12	-2.25	3.0
	2.300		5.5	-2.70	2.7
	Unirradiated	-3.0	144		
	control	-3.5	70		
		-4.0	42		
		-4.5	19		
Exp. 5. BSV on <i>N. glutinosa</i>					
0.1	0.45	-2.3	92	-0.73	3.7
	0.77		68	-0.95	2.8
	1.08		31	-1.53	3.3
	1.40		25	-1.73	2.8
	Unirradiated	-3.0	95		
	control	-3.5	47.5		
		-4.0	26.5		
		-4.5	16		

* r is the proportion of residual activity of irradiated virus preparations. The values of $\log_{10} r$ were obtained by graphic interpolation from the dilution curve given by unirradiated virus.

$\dagger k$ is obtained from the equation $\log_{10} r = -0.4343kt$, where t is time of irradiation in minutes.

(Latarjet & Wahl, 1945, Latarjet & Morenne, 1951; Kleczkowski & Kleczkowski, 1953). This is insufficient evidence to conclude that single quanta of ultraviolet inactivate, and the minute quantum yield, of the order of 4×10^{-5} (Oster & McLaren, 1950), tells against such a conclusion. Inactivation of many biologically active particles by heat also approximates to a first-order reaction, but no one suggests that heat inactivation occurs because of a 'single hit'. Approximation to a first-order reaction means simply that, in constant conditions, there is a constant probability, $p = 1 - e^{-k}$, that a given particle which is still active will become inactive during the next minute.

There is evidence, too, that absorption of ultraviolet can lead to different kinds of changes. With the plant viruses we have noticed only two, loss of infectivity and the production of particles that can infect plants exposed to visible light but not those kept in the dark. With some *Rhizobium* phages (Kleczkowski & Kleczkowski, 1953) and trypsin (to be published), there is evidence of a third change, for the particles remaining active after irradiation are less stable than particles that have not been exposed to ultraviolet radiation. We have failed to detect such an effect during comparable tests with the three plant viruses, but their greater intrinsic stability than either of the bacteriophages or of trypsin may explain the failure. Preparations of the three viruses, irradiated so that their residual infectivity was about 0.5 % of control preparations when tested immediately after irradiation, had the same relative infectivity when again compared with the controls after a week at 18°. This treatment had no obvious effect on the activity of control preparations and any increase in the rate of spontaneous inactivation produced by irradiation would of necessity have had to be considerable to be detected. That irradiation may affect stability is suggested by the increased sensitivity of irradiated preparations of TMV to denaturation by heat (Oster & McLaren, 1950).

Preparations of BSV consist of spherical particles of a uniform size, and those of RTNV of spherical particles of two different sizes, whereas preparations of TMV consist of rod-shaped particles of greatly differing lengths. It is of some interest, therefore, that inactivation of all three viruses should follow the same course. Despite the differences in their tendencies to aggregate, it seems that particles of all three viruses behave as single infective units and not as aggregates of units all of which need to be inactivated individually. If they consisted of such aggregates, the constant probability, p , of inactivation, would be that of single units and not of aggregates; the probability of inactivation during the next minute of an aggregate containing n active units would be p^n , and this would increase as n decreases during the course of irradiation. The infective units in preparations of TMV, then, are not predominantly aggregates containing more than one infective unit. The small particles have been shown to be poorly infective and not to gain infectivity when aggregated linearly (Bawden & Pirie 1945), and it seems probable that few of the large aggregates contain more than one infective unit.

The experiments recorded in Table 3 were made at different times and with plants kept under different light intensities after inoculation, so that no exact

conclusions should be drawn from quantitative differences between values recorded for k . However, the large effect of varying virus concentration is obvious. The higher the concentration, the lower is the relative rate of inactivation, showing that increasing virus concentration produces a proportionately smaller increase in the amount of ultraviolet absorbed, so that less radiation is absorbed per virus particle in a unit of time.

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(Received 7 August 1952)

FULTON, J. D. & RIMINGTON, C. (1953). *J. gen. Microbiol.* 8, 157-159.

The Pigment of the Malaria Parasite *Plasmodium berghei*

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SUMMARY: The pigment of *Plasmodium berghei*, a recently discovered parasite of a wild rodent, *Thamnomys surdaster*, in the Belgian Congo, has been isolated from the infected red cells of young rats in which the strain is maintained in the laboratory. It has been shown by chemical and spectroscopical evidence to be haematin.

Early work by Brown (1911) indicated that the pigment occurring in the spleen and liver of malarial patients infected with *Plasmodium falciparum*, the parasite of malignant tertian malaria, was haematin. Sinton & Ghosh (1934*a, b*) and Ghosh & Sinton (1934) extracted the pigment from the red cells of monkeys heavily parasitized with *P. knowlesi* and established that this substance was closely similar to or identical with haematin. Devine & Fulton (1941) and Morrison & Anderson (1942) presented further data in support of the nature of this monkey pigment. From spectroscopic and other evidence, that present in *P. gallinaceum* of chickens was also shown by Devine & Fulton (1942) to be haematin. Because the above authors used dilute alkali for extraction, which is known to bring about changes in the properties of this substance, Rimington & Fulton (1947) reinvestigated the pigment of *P. knowlesi* and of *P. gallinaceum* using concentrated phenol for extraction, and established beyond reasonable doubt that the vinyl side chains in the haematin remained unaffected. Recently a plasmodium, which parasitizes the wild rodent *Thamnomys surdaster* in the Congo, has been described by Vincke & Lips (1948) and named by them *Plasmodium berghei*. It readily infects mice, rats, hamsters and other laboratory animals, and has proved useful in chemotherapeutic experiments. This note describes a chemical investigation of the pigment present within the parasite.

Experimental

Hooded rats of weight 60-80 g. were inoculated intraperitoneally with infected blood from a donor rat and 1 week later, when the infection had reached a peak, the animals were anaesthetized and then bled by cardiac puncture in presence of heparin. The infected and non-infected red cells obtained by centrifugation were laked in distilled water and repeatedly washed till free from haemoglobin. The residue was kept under ethanol till sufficient was available for extraction of pigment. This extraction was carried out as described by Rimington & Fulton (1947), using a concentrated solution of phenol prepared by adding 10 ml. water to 90 g. of crystalline phenol. The pigment-containing material was mixed at room temperature with twice its

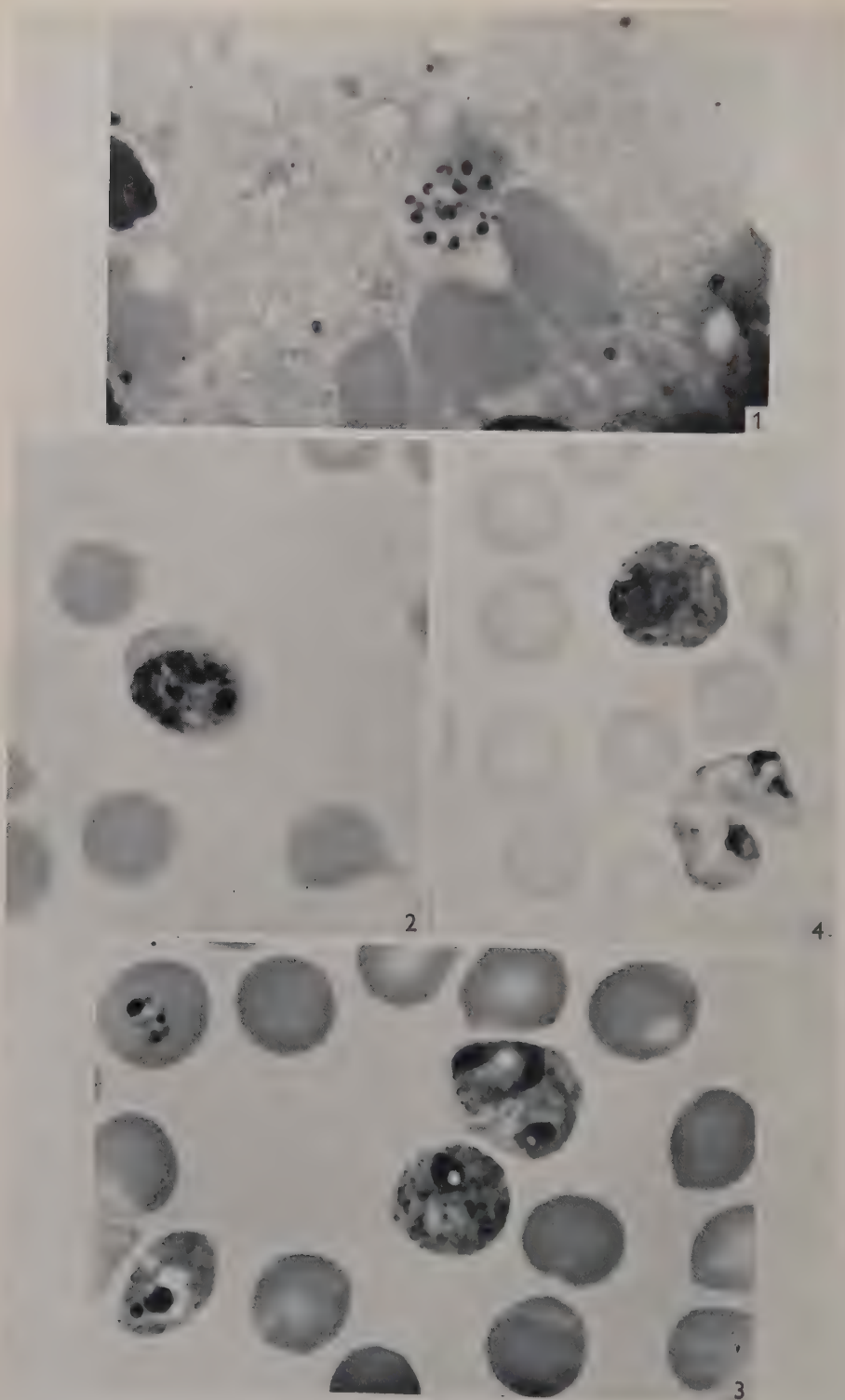
volume of the 90 % phenol solution, and was frequently shaken during a period of several hours. The process of extraction was repeated till the absorption band at 627 m μ . was faint or absent. The combined phenol extracts were then mixed with twice their volume of ethanol and filtered through a no. 4 sintered glass funnel to remove precipitated protein. The solution was then dialysed against tap water in a cellophan bag and the contents, which separate into two layers, were frequently mixed. Some stringy material appeared at this time but contained only small amounts of pigment and was not further investigated. Dialysis was continued against distilled water till turbidity occurred in the coloured layer or till its volume was markedly decreased. On mixing this coloured layer with a large volume of water a fine precipitate was produced. The latter was collected by centrifugation and washed repeatedly in distilled water and finally dried *in vacuo*. It was noteworthy that the washed red cells containing *P. berghei* were very much paler than a corresponding volume of material containing *P. knowlesi* or *P. gallinaceum* and furnished much less pigment than the last two samples. Difficulty is frequently experienced in observing the pigment of some strains of *P. berghei* in stained smears of blood, but in that at present employed it was more abundant and as stated by the original authors 'on peut distinguer dans le cytoplasme des grains très fins d'un pigment noir'. In gametocytes it occurs in smaller grains and is more widely scattered as shown in Pl. 1. The photomicrographs of the malarial parasites were recorded on Barnet Orthochromatic plates, employing a Leitz 2 mm. 1.4 N.A. oil immersion apochromatic objective, and a Leitz projection ocular. A Wratten no. 45 filter was used to obtain maximum contrast.

Chemical examination of the residue after dialysis

A small quantity of the residue was dissolved in 0.01 N-NaOH and one-fifth of its volume of pyridine was added followed by a little sodium dithionite. The spectrum of pyridine haemochromogen appeared (band 557 m μ .). The remainder of the residue was subjected to the Grinstein (1947) procedure for the isolation of protoporphyrin ester from haemoproteins. The crystals obtained had m.p. 219° (uncorr.) and absorption maxima in chloroform 631.2, 576.7, 540.4, 508.9 m μ . and appeared to be identical in all respects with protoporphyrin IX dimethyl ester. The original pigment is thus identified as haematin.

Comment

The amount, appearance and location of pigment in different species of malaria parasites and in different developmental forms of the same parasite show wide variations. The effect on the host cell also differs. In human infections the enlarged pale cell in *P. vivax* infections in which fine yellowish brown pigment is formed contrasts with the normal appearance of the red cell and the presence of abundant coarse dark pigment in infections with *P. malariae*. Because of the apparent anomaly in the case of the latter parasite regarding the appearance of the red cell and the amount of pigment formed, some authors have found it difficult to accept that the pigment arises from the



J. D. FULTON & C. RIMINGTON—MALARIA PIGMENT. PLATE 1

haemoglobin of the host cell. In some strains of *P. berghei* the pigment is frequently difficult to detect; the original authors described it as dark and in very fine granules. In certain monkey and fowl parasites the pigment is characteristic. In all malaria parasites so far examined the material has been shown to be haematin.

Acknowledgement is made to Dr J. E. Falk for help in isolation of the protoporphyrin, and to Mr M. R. Young for the photomicrographs.

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EXPLANATION OF PLATE

- Fig. 1. Schizont of *P. berghei* from hamster bone marrow showing deeply stained chromatin and large granules of lighter pigment scattered throughout the cytoplasm. $\times 2250$.
- Fig. 2. Schizont of *P. berghei* from rat blood showing finer pigment granules than that from hamster. $\times 2250$.
- Fig. 3. Female gametocyte of *P. berghei* from hamster blood showing abundant fine pigment granules and small peripheral nucleus. Three other infected cells present. $\times 2250$.
- Fig. 4. Male gametocyte from rat blood with abundant pigment granules throughout the cytoplasm and large deeply stained nucleus. A doubly infected red cell is shown below it. $\times 2250$.

(Received 17 July 1952)

Lactobacillus malefermentans n.sp., Isolated from Beer

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SUMMARY: A new species of heterofermentative *Lactobacillus* has been isolated from top-fermentation beer in which it occurs as a contaminant. The organism is distinguished principally by the fact that it ferments only glucose, maltose and inulin and, in consequence, it has been designated *L. malefermentans*.

During studies of lactic acid bacteria which infect brewery yeast and beer a rod-shaped organism which was isolated showed marked differences from other species of *Lactobacillus* described in the literature. Details of the isolation have already been published by Walker & Parker (1943) and the organism was designated provisionally D2. A description of its characters and behaviour is now given.

METHODS

The behaviour of the organism was studied in liquid and in solid media prepared from beer, both hopped and unhopped, and from yeast-extract and peptone. Unlike many types of lactic acid bacteria which are found in beer the new organism was unable to proliferate in unhopped brewery wort. The action of the bacterium on carbohydrates was studied in a casein double digest prepared according to the directions of Davis (1939), and supplemented by addition of yeast autolysate (1 ml./100 ml. digest).

DESCRIPTION OF THE ORGANISM

Morphological characters

Shape, size and arrangement of cells. In unhopped beer after 24 hr. at 30°, the majority of the cells were 2-6 μ . long and all were rod-shaped. Single cells, pairs and a few short chains were noted. Motility was not observed.

Staining. The cells were Gram-positive. Neither endospores nor flagella were detected and attempts to detect capsules by the use of several staining procedures were unsuccessful.

Cultural characters

Growth on solid media, in CO₂ at 25°. On a beer agar slope at 4 days, moderately strong growth, beaded to nodose; the colonies being greyish, glistening and mostly about 1-2 mm. in diameter. On a yeast-extract peptone glucose (YEPG) agar slope at 7 days moderately strong nodose growth, the colonies being of irregular shape, white and opaque. On nutrient agar slope slight nodose growth.

Stab. In YEPG agar at 25° for 5 days growth was strong and filiform and did not occur at the surface. In YEPG gelatin at room temperature during 5 days filiform growth developed without beading or branching.

Growth in unhopped beer at 30°. A slight deposit was noted at 3 days, and on shaking the tube this produced a uniform billowy turbidity.

Growth in YEPG broth at 25°. At 2 days a moderate deposit had collected and gave a non-silky turbidity when shaken.

Growth in Lemco peptone broth in CO₂ at 25°. At 6 days a scanty powdery sediment had collected and the liquid was clear.

Physiological characters

Relation to temperature: in unhopped beer the optimum range for growth is 25–34° with a minimum temperature of 10° and a maximum temperature of 37°. Relation to oxygen: facultative anaerobe. Relation to hydrogen-ion concentration: in unhopped beer the optimum pH value is 4.3 with limits 4.1 and 6.9. Resistance to heat: killed by heating for 15 min. at 60–65° in unhopped beer. Resistance to hop antiseptic, markedly restricted.

Biochemical characters

Catalase reaction, negative. Formation of acetylmethylcarbinol, none. Nitrate reduction, none. Indole formation, none. Gelatin liquefaction, none. Action on litmus milk, no change.

Carbohydrates utilized. In a casein double digest medium supplemented with yeast autolysate the organism strongly attacked glucose, maltose and inulin, in all cases with production of acid, but no gas collected in Durham tubes. Dulcitol was attacked slightly. Inulin yielded more acid than did glucose. No other carbohydrates were effected by the organism.

Nature of the acid produced from glucose. Lactic acid was estimated by conversion to acetaldehyde after deproteinization of the medium with copper sulphate and calcium hydroxide and, after similar deproteinization of another sample of the medium, acetic acid was estimated after distillation in the presence of sulphuric acid. The molecular ratio of lactic acid to acetic acid in the medium was found to be 100:38. In a separate experiment the lactic acid was isolated as the zinc salt by the method of Pederson, Peterson & Fred (1926) and was optically inactive.

CLASSIFICATION

The characters—a non-motile, non-sporing, Gram-positive rod, which is catalase-negative, facultatively anaerobic, does not reduce nitrate and ferments carbohydrates—places the organism in the tribe Lactobacilleae Winslow *et al.* of the family Lactobacteriaceae Orla-Jensen. The production of large amounts of lactic acid causes it to be assigned to the genus *Lactobacillus* Beijerinck. As to its specific characters the organism has been compared with species of *Lactobacillus* already described in the literature (Pederson, 1938; Orla-Jensen, 1942; *Bergey's Manual*, 1948; Shimwell, 1949). On the evidence it has been concluded that *L. pastorianus* van Laer is the only *Lactobacillus* species which shows in some respects attributes similar to D2. The latter usually is seen as rods about 2 μ . in length though forms up to 6 μ . in length may occur, whereas *L. pastorianus* is normally of length 5–10 μ . while filamentous forms up to

35 μ . in length have been noted. *L. pastorianus* shows beaded to arborescent growth in gelatin stabs, while D2 produces definitely a filiform growth in gelatin. Further, *L. pastorianus* produces acid in milk while D2 is unable to do so. Finally, *L. pastorianus* ferments arabinose, glucose, fructose, galactose, maltose and sucrose, while D2 can strongly attack only glucose, maltose and inulin, and exercise a weak action on dulcitol. Orla-Jensen (1942) has remarked that true lactic acid bacteria rarely attack dulcitol and then only slightly. It is also of interest to record that D2 attacks inulin even more vigorously than it attacks glucose, unexpected behaviour in view of the inability of this organism to assimilate fructose. These differences are deemed sufficiently large to justify species differentiation and, accordingly, we formally propose D2 as a new species under the designation *L. malefermentans* (male = peculiarly, fermentans = fermenting) which name has been chosen in view of the inability of the organism to ferment arabinose, xylose, fructose, galactose and sucrose and, on the other hand, of its markedly vigorous attack on inulin.

Lactobacillus malefermentans n.sp. Rods 2–6 μ . in length, occurring principally as single cells and pairs. Non-motile, no endospores, Capsules and flagella not detected. Gram-positive. Colonies on beer agar beaded to nodose, greyish, glistening, 1–2 mm. diameter.

Facultative anaerobe. Optimum temperature 25–34°, minimum 10°, maximum 37°. Optimum pH value 4.3. Grows vigorously in unhopped beer and in yeast-extract peptone glucose broth. No growth in brewers' unhopped wort (a malt-extract solution). Attacks glucose, maltose and inulin with production of acid but not of gas. Dulcitol fermented weakly. Heterofermentative in glucose media, the lactic acid formed being optically inactive. Isolated at Manchester from sour beer, March 1942.

Subcultures from the type culture have been deposited at the National Collection of Industrial Bacteria, The Chemical Laboratory, Department of Scientific and Industrial Research, Teddington, Middlesex, (Culture No. NCIB 8517), at the National Institute for Research in Dairying, Shinfield, Reading, and at the Laboratories of the Brewing Industry Research Foundation, Nutfield, Surrey.

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(Received 30 July 1952)

MANSON, E. E. D. & POLLOCK, M. R. (1953). *J. gen. Microbiol.* 8, 163-167.

The Thermostability of Penicillinase

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SUMMARY: Extracellular penicillinase produced by the growth of five different strains of the genus *Bacillus* in casein hydrolysate is rapidly inactivated at 100°, but can be strikingly protected against such inactivation by addition of 1 % gelatin or high molecular weight substances present in tryptic digest and meat infusion broth.

Since Abraham & Chain (1940) first described penicillinase there have been conflicting reports on the thermostability of the enzyme. Most workers (see Florey, Chain, Heatley, Jennings, Sanders, Abraham & Florey, 1949) have reported rapid destruction on heating above 50° in aqueous solution at neutral pH. Duthie (1944), however, showed that penicillinase in the filtrate of a broth culture of *Bacillus subtilis* was unaffected by heating at 100° for 20 min. at pH 7.0, and Smith & Smith (1945) reported that penicillinase from a strain of paracolon bacillus was stable at 100° for 30 min. when dry, but was completely inactivated in aqueous solution. In view of these reports it was decided to test a number of penicillinase preparations from different bacterial cultures of the genus *Bacillus* to discover whether their thermostability was determined by the strain of organism used.

METHODS

Media and materials. Casein hydrolysate medium (CH) was made from acid hydrolysed vitamin-free casein (Allen and Hanbury's Ltd.) 0.8 % (w/v) supplemented with 8×10^{-5} M-L-tryptophan, 2×10^{-4} M-DL-cystine, 1.7×10^{-3} M-MgSO₄ and 0.02 M-phosphate buffer, pH 7.0.

Infusion broth was prepared as described by Wright (1933), but using only a quarter of the amount of water, to give a concentrated medium. Tryptic digest broth was prepared as described by Hartley (1922).

The penicillin used was sodium benzylpenicillin from Glaxo Laboratories Ltd. The gelatin solutions were prepared from 'Difco' standardized gelatin.

Five commercial penicillinase preparations (A-D and F) and the strains used in their production, with the exception of strain F, were obtained. These commercial strains were called (A) *B. licheniformis*, (B) *B. cereus*, (C) *B. subtilis* and (D) *B. subtilis* and were used for the laboratory penicillinase preparations together with a penicillinase-producing strain of *B. cereus* (NRRL 569 designated here as E).

Enzyme production. Washed cell suspensions were prepared from CCY medium (Gladstone & Fildes, 1940) (organisms A-D) or tryptic digest broth (organism E) after incubation for 18-24 hr. at 35° on a bacteriological shaker. The dry weights were determined by opacity measurements on the 'Spekker' absorptiometer by reference to a standard curve of the relation between

opacity and dry weight. Cell suspensions at an initial concentration of 1 mg./ml. were incubated in 50 ml. CH medium or tryptic digest broth in a 250 ml. conical flask and penicillin (1–10 units/ml.) was added to increase the yield of enzyme. These cultures were shaken for 3–3½ hr. at 35° before the cells were centrifuged off. The supernatant fluids (having penicillinase activities from 200 to 1000 μ l. CO₂/hr./ml.) were stored at 2°.

Gelatinase activity. Strains were tested for gelatinase activity by incubating 'stab' cultures in broth containing 15% (w/v) gelatin for 3 weeks at 18°; a positive result was recorded if any liquefaction had occurred. The culture supernatant fluids were similarly tested, after sterilization by filtration, by incubating 0.5 ml. on the surface of 2 ml. of the gelatin-broth in a test-tube at 18° for 5 days.

Penicillinase assay. The enzyme was assayed manometrically at pH 7.0 and 30° by the method of Henry & Housewright (1947), and the penicillinase activity expressed in μ l. CO₂ liberated/hr./ml. of medium. No corrections have been made for the small amounts of CO₂ retention in the different media, which will cause a slight error in the enzyme activity values. The flask constants did not vary enough for such retention to affect comparative results.

Heat stability test. A 250 ml. flask with a stoppered side arm was connected to a water cooled reflux condenser and placed in a boiling water-bath. Through the side arm, so made that a pipette passing through it could reach the base of the flask, approximately 8 ml. of enzyme solution were added rapidly. Samples of 2 ml. were removed at intervals and immediately placed in test-tubes, precooled in a beaker of ice, and kept cold until assayed.

RESULTS

The thermostability of the five commercial preparations was examined; three (A–C) were found to be fairly stable at 100°, whereas the other two (D, F) were rapidly inactivated (see Table 1).

Further studies were made of the thermostability of penicillinase from four of the organisms concerned (A–D) and also organism (E).

Gelatin. Enzyme produced in CH medium was found to be rapidly inactivated at 100° irrespective of the bacterial strain used or stability of the commercial preparation; but this inactivation was markedly reduced if 1% (w/v) gelatin was added to the enzyme preparation before heat treatment. Enzyme produced in a 'complex' medium, e.g. tryptic digest broth, was inactivated only slightly at 100° and to about the same extent as CH preparations containing 1% gelatin (Table 1 and Fig. 1). The concentration of gelatin which gave maximum protection was found to be 1% when a range of concentrations from 0.01 to 2.5% (w/v) was tested (Table 2). A twofold difference in concentration of enzyme (from growth of E in CH) did not affect the extent of protection afforded by 1% gelatin.

The addition of 1 and 2.5% gelatin to the commercial preparation (F) had no protective effect on the enzyme which was still completely inactivated after 2 min. at 100°.

Table 1. *Thermostability of penicillinase preparations*

Organism	Growth medium	Commercial preparation		Laboratory preparation			Time of heating (min.)
		Activity (μ l. CO ₂ /hr./ml.)	Residual activity (%)	Casein hydro-lysate -gelatin (%)	Casein hydro-lysate +gelatin* (%)	Hartley broth -gelatin (%)	
A. <i>B. licheniformis</i>	Papain digest 1:3 distilled water + 0.5% glucose	1080	—	38	92	—	2
			—	38	88	—	5
			46	—	—	—	15
			20	—	—	—	60
B. <i>B. cereus</i>	Difco yeast beef broth	4500	—	19	84	87	2
			—	16	80	83	5
			80	—	—	87	15
			27	—	—	—	60
C. <i>B. subtilis</i>	Tryptic digest broth + 1% glucose	600	90	<0.1	75	—	2
			85	<0.1	70	—	5
			73	—	—	—	15
			37	—	—	—	60
D. <i>B. subtilis</i>	Lemco broth	700	4	22	79	84	2
			<0.1	13	73	74	5
			<0.1	—	—	65	15
			<0.1	—	—	—	60
E. <i>B. cereus</i> † (NRRL 569)	—	—	—	19	48	53	2
			—	14	42	47	5
			—	—	—	43	15
			—	—	—	—	60
F. Unknown†	Unknown	340	<0.1	—	—	—	2
			<0.1	—	—	—	5

Residual activity figures represent percentage of original activity remaining after heating at 100° for periods stated.

* 1% gelatin added to enzyme preparation.

† Positive gelatinase activity.

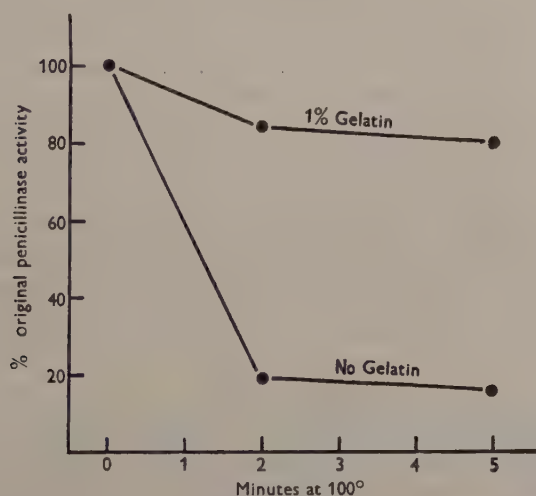


Fig. 1. Protective effect of 1% gelatin against inactivation of penicillinase at 100°. Enzyme was the supernatant fluid from a culture of organism B in casein hydrolysate.

Concentrated infusion broth. The addition of concentrated infusion broth to an equal volume of the enzyme preparation had the same protective effect as 1 % gelatin. Concentrated broth (25 ml.), when dialysed against glass-distilled water (4.5 l.) at 2°, suffered no loss in protective ability (Table 2).

Table 2. *Effect of added substances on heat stability of penicillinase from organism B grown in casein hydrolysate*

Exp.	Added substance	Percentage original activity remaining after treatment at 100° for	
		2 min.	5 min.
I	Nil	7	4
	Infusion broth	71	69
	Infusion broth after dialysis for 24 hr.	71	—
II	Nil	19	16
	0.01 % gelatin	42	39
	0.05 % gelatin	55	56
	0.1 % gelatin	65	66
	1.0 % gelatin	84	80
	2.5 % gelatin	84	76
III	Nil	11	17
	5×10^{-3} M penicillin	56	50
	5×10^{-3} M penicillin	76	76
	5×10^{-3} M penicilloic acid	28	25
	5×10^{-3} M penicilloic acid	38	46

Penicillin, added to the enzyme solution cooled to 0° immediately before heating at 100°, protected the enzyme to some extent, whereas an equivalent concentration of the product of enzymic hydrolysis (penicilloic acid) had only a slight effect on the thermostability (Table 2). The penicilloic acid was prepared by incubating the penicillin and enzyme together at 30° before treatment. The greater part of the enzyme prepared as described in 'Methods' was formed adaptively, following induction by addition of the substrate. This preparation was compared with that of 'basal' enzyme produced without added penicillin, but no difference in thermostability could be detected (Manson & Pollock, to be published).

Miscellaneous. 8-Hydroxyquinoline at the concentration (8.8×10^{-4} M) used for inhibiting adaptation had no effect on thermostability, but another chelating agent ethylenediamine tetra-acetic acid ('Versene', Bersworth Chemical Co., Framingham, Mass.) in high concentrations (0.005 and 0.5 M) and gum acacia 1 % (w/v) each had slight protective properties.

DISCUSSION

The results show that the penicillinase in these preparations is rapidly inactivated in aqueous solution at 100°, but can be strikingly protected by the addition of gelatin or some high molecular weight compounds present in complex media. A similar observation with pectic enzymes has been made by

Matus (1948), who found that gelatin and other compounds gave some protection against heat inactivation up to 60°. This effect was shown by penicillinase from four of the five strains tested and to a lesser extent by that from the fifth strain (E). It thus appears that the heat stability of penicillinase in crude preparations depends on the other constituents present during heat treatment, rather than on the bacterial source. The decreased protective action of gelatin on the penicillinase from strain E and its inability to protect enzyme in commercial preparation F, may be partly due to destruction of the gelatin by the gelatinase known to be present in both these cases.

It is possible that the protective effect of gelatin is due to its combination with the enzyme to form a heat stable complex. This is indeed suggested by the time curves for penicillinase inactivation shown in Tables 1 and 2, where it can be seen that little further enzyme was destroyed by prolonging heat treatment from 2 to 5 min. The immediate loss in 2 min. might thus be due to inactivation of the labile unassociated enzyme and the slow subsequent loss to that of the fraction stabilized by combination with gelatin or other protecting substance. The stabilizing action of a substrate on its enzyme is well known, and the protective effect of penicillin against destruction of penicillinase at 100° is almost certainly due to the formation of a relatively stable enzyme-substrate complex.

We wish to thank the various manufacturers concerned for their co-operation in sending us samples of penicillinase, details of techniques and media employed in their preparation, and subcultures of bacterial strains used.

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(Received 30 July 1952)

The Occurrence of Independent Mutations to Different Types of Streptomycin Resistance in *Bacterium coli*

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SUMMARY: Streptomycin resistant strains of *Bacterium coli* were obtained by plating numerous 1 or 1.5 ml. volume cultures of a sensitive strain on plates containing 100 μg . streptomycin/ml. The growth rate of these strains was measured by mixing them with the sensitive strain and measuring the fall in the numbers of resistant organisms during several periods of daily subculture in streptomycin free broth. Their resistance to streptomycin and stability during successive subcultures in broth were also studied. They could be divided into three main types. All type I strains had identical slow growth rates, were completely resistant, though growing poorly at high streptomycin concentrations and were quickly replaced by rapidly growing slightly resistant variants when subcultured in broth. All type II strains had identical slightly more rapid growth rates, were partially resistant and gradually decreased their resistance in a stepwise manner during subculture. Type III strains had growth rates slightly less than that of the sensitive strain and of a degree of variability similar to those found in clones of the sensitive strain. They were completely resistant and were stable during subculture. They could be subdivided on the basis of one characteristic. Type IIIa strains grew as well in the presence or absence of streptomycin but type IIIb strains grew much more slowly in the presence of 1600 μg . ml. streptomycin or over. Since all the strains from any one plate were usually of the same type they were considered to have arisen from a single mutant cell. Since each mutation occurred several times in independent cultures and the number of tubes in which multiple mutations occurred was not greater than would be expected by chance, each type is considered to have arisen by an independent mutation.

Bacterial populations sensitive to streptomycin have been shown to contain small numbers of resistant organisms. Using *Bacterium coli* strain B/r, Newcombe & Hawirko (1949) studied the number of organisms growing in plates containing graded concentrations of streptomycin and found two types of resistant strains. One of these was 2-4 times less sensitive than the parent strain, which was inhibited by about 8 μg ./ml. The other type, obtained from plates containing concentrations of streptomycin above 16 μg ./ml., was capable of growth in concentrations of at least 1000 μg ./ml. Meads & Haslam (1949) obtained similar results with *Bact. friedlaenderi*. Alexander & Leidy (1947) found no difference between the number of colonies of *Haemophilus influenzae* which appeared when a given suspension was seeded in media in the presence of 100 and 1000 μg ./ml.

In the course of experiments with streptomycin resistant strains of *Bact. coli* obtained by plating out a sensitive strain in the presence of 100 μg ./ml. it became clear that resistant strains could be divided into a number of types. The purpose of this paper is to show that in such strains four distinct types of

resistant organisms may be recognized in addition to dependent organisms, and that each arises by independent mutations. The strains have been studied from the point of view of growth rate, degree and type of resistance to streptomycin, and stability during successive cultures in medium free of streptomycin. In addition, it will be shown that strains adapted to streptomycin by multiple steps differ from any of these types, though most closely resembling one of them.

METHODS

Strain of Bacterium coli. A typical strain of *Bact. coli* var. *communis* was used, inhibited by a streptomycin concentration of about 5 $\mu\text{g.}/\text{ml.}$ A Dorset's egg slope was inoculated from a single colony and, after incubation, kept in the refrigerator. An 18 hr. broth culture inoculated from this was used for the inoculum in all the experiments.

Medium. The medium used was a modified Hartley's digest broth containing tryptic digest of beef and adjusted to pH 7.5. Solid media contained 1.5 % (w/v) agar.

Procedure. A series of tubes containing 1 ml. of broth was inoculated with one loopful of an 18 hr. broth culture diluted so that each loopful contained 50–200 organisms. After incubation at 37° for 18–24 hr. the contents of each tube were spread over the surface of an overdried nutrient agar plate containing 100 $\mu\text{g.}$ streptomycin/ml., a few of the tubes being reserved for a viable count. The plates were incubated for 48 hr. Any colonies which grew were counted and each colony was subcultured (*a*) on to an agar slope or a Dorset's egg slope which was kept in the refrigerator as a stock culture, (*b*) on to a portion of a streptomycin ditch plate in order to distinguish between resistant and dependent strains, and (*c*) into a tube of plain broth which was incubated for 18 hr. and then used for determining the growth rate. Strains derived from colonies from the same plate which had similar characteristics were said to be derived from the same resistant clone. Dependent strains were discarded.

In some experiments a 50 ml. volume of broth was also inoculated with the sensitive strain. After incubation the centrifuged deposit was divided between a number of streptomycin plates, and the colonies from one of these were investigated in a similar manner.

Viable counting. A dropping pipette calibrated by weighing to deliver 0.02 ml./drop and a single 3 mm. loop calibrated by weighing to remove 0.0044 ml./loopful were used. With a pipette 0.5 ml. of culture was added to 9.5 ml. of diluent (1 % broth in distilled water). From this two 0.02 ml. drops were added to bottles containing 0.96, 10 and 100 ml. diluent. From the appropriate dilutions a loopful was spread over a quarter of the surface of a plate. With rapidly growing strains the plates were incubated for 18 hr. at 30°, whereas, with slowly growing strains, they were incubated at 37°. In this way 200–300 colonies could easily be counted on each segment of a plate. A fully grown broth culture usually yielded 100 colonies from the highest dilution. In a series of duplicate counts from such cultures the coefficient of variation was *c.* 11 %, the theoretical figure from the number of colonies

counted being 9%. The pipette and the calibrated dropper were sterilized between counts by pipetting boiling water in and out several times. Using this method a count could be done rapidly and with few materials.

Growth rates. An indirect method, which has not been previously described, was used for determining growth rates. Equal volumes of the first broth subculture from a resistant colony and of a similar broth culture of the sensitive strain were mixed. From this mixture a viable count was done on plates with

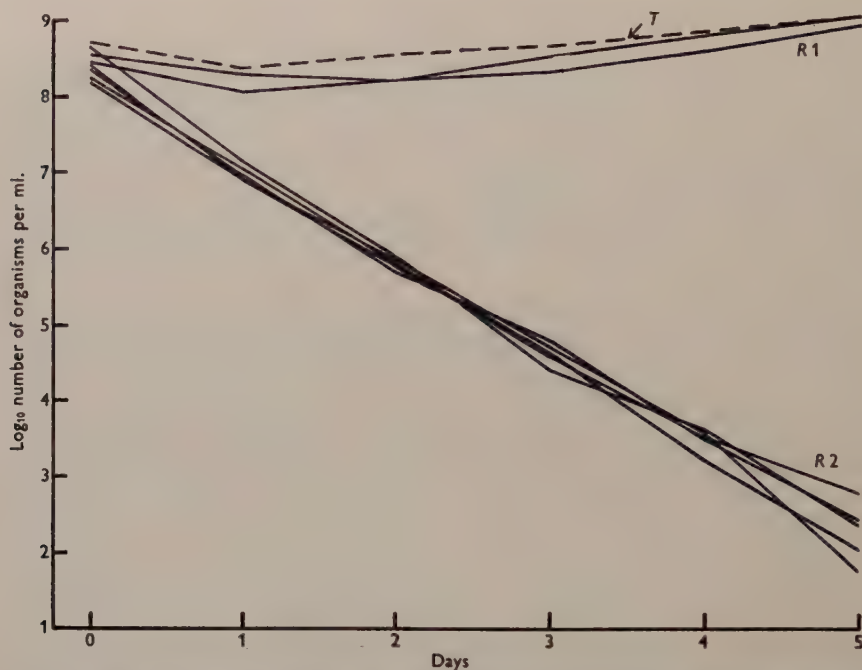


Fig. 1. *T*, total count. *R1*, group of two resistant strains growing at the same rate as the sensitive strain. *R2*, group of nine resistant strains of which only five are represented for the sake of clarity, growing at a slower rate than the sensitive strain.

and without streptomycin 100 $\mu\text{g.}/\text{ml.}$, so that the total number of organisms and the number of resistant organisms was known. A standard 3 mm. loopful (0.005 ml.) of the mixture was also subcultured into a tube of broth. After 24 hr. incubation a differential viable count was made from the second tube and the mixed growth was subcultured with the same loop into a fresh tube of broth. This procedure was repeated at daily intervals. In most of the experiments counts were done on the initial mixture and on the mixture after either one and two or two and four daily subcultures according to the rapidity with which the resistant strain was eliminated. The same batch of broth was used throughout any one experiment.

In Fig. 1 an experiment illustrating the method is shown in which two clones of resistant strains having growth rates the same and much slower than the sensitive strain were tested simultaneously. For any of these strains there

was a straight-line relationship between the number of resistant organisms found in the mixture and the number of successive daily subcultures. The linearity of the relationship was proved in this and other experiments by an analysis of variance. The slope of this line, g_I , was used as a measure of the differential growth rate.

The relationship between g_I and the increase in numbers of the resistant and sensitive strains, during their logarithmic phases of growth, g_r and g_s , can be seen from consideration of Fig. 2.

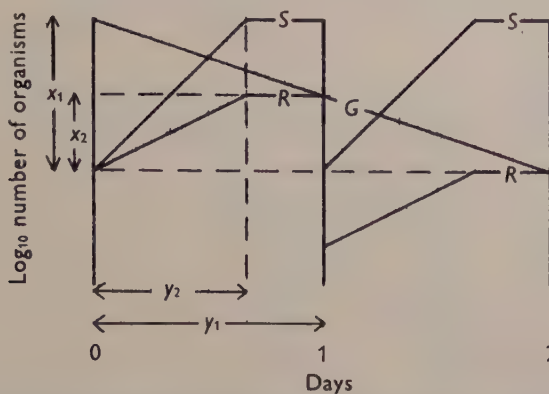


Fig. 2. Diagrammatic representation of the growth of a mixture of a sensitive and a resistant strain during two successive subcultures. S , growth curve of sensitive strain; R , growth curve of resistant strain; G , line representing the fall in the numbers of the resistant strain; x_1 , the increase in concentration of the sensitive strain during a 24 hr. period of culture; x_2 , the increase in concentration of the resistant strain during the same period; y_1 , the duration of a period of culture = 1 day; y_2 , the period during which growth takes place.

Let g_s = growth rate of the sensitive strain; g_r = growth rate of the resistant strain; g_I = slope of the line relating fall in number of resistant organisms to period of subculture in days. This line corresponds to those illustrated in Fig. 1. Then

$$g_s = \frac{x_1}{y_2}, \quad g_r = \frac{x_2}{y_2}, \quad g_I = -\frac{x_1 - x_2}{y_1},$$

x_2 and y_2 are eliminated from these equations, and $y_1 = 1$, so that:

$$\frac{g_r}{g_s} = \frac{x_1 + g_I}{x_1}. \quad (1)$$

Assuming that the growth rate of the sensitive strain is constant, and known, the growth rate of the resistant strain can be calculated from g_I if the logarithm of the ratio between the volume of the inoculum and the volume of the tube of broth (x_1) is determined. This assumes that the final concentration of the organisms after each growth period is the same.

Viable counts on plates with and without streptomycin were done at hourly intervals during the growth of a mixture of the sensitive and a resistant strain. The form of these curves, shown in Fig. 3, corresponds to the diagrammatic representation in Fig. 2.

The indirect method of measuring growth rates is particularly well suited to measure small differences in growth rates between several strains. It was found possible to investigate up to forty strains simultaneously. Any variations in the experimental conditions, such as the final concentration in the cultures, affected each strain to the same extent and did not destroy the validity of the comparison.

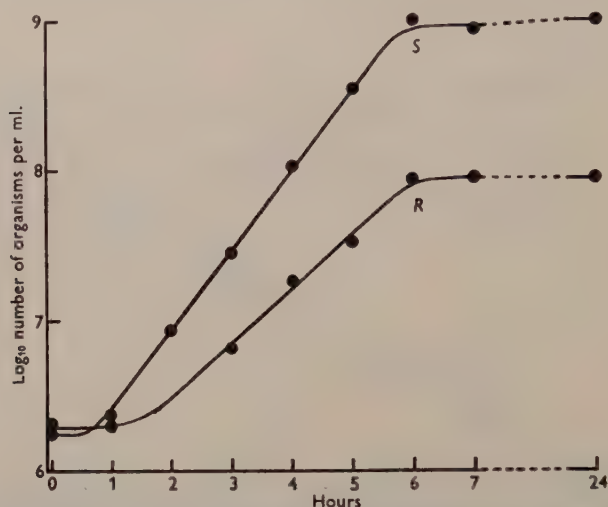


Fig. 3. Growth curves of the sensitive and a resistant strain growing together in broth. *S*, sensitive strain; *R*, resistant strain.

As a check on this method viable counts were done at hourly intervals during the logarithmic phase of growth on a series of five strains, including the sensitive strain. The indirect method was done at the same time in the same batch of broth. It was possible to compare the growth rates obtained by measuring the slopes of the logarithmic portion of the growth curves with the estimates of these growth rates obtained from the values of the differential growth rates (indirect method) by the use of equation (1). The results are given in table 1. There was no significant difference between the two estimates for each resistant strain. The indirect method yielded values of g_I from 0 to -1.24 , having standard errors of ± 0.045 to ± 0.090 . It was slightly more accurate than the direct method which gave values of g_s or g_r from 0.845 to 0.463 with standard errors of ± 0.059 to ± 0.032 .

Streptomycin sensitivity. Where a large number of strains was being tested simultaneously nutrient agar plates were prepared containing concentrations of streptomycin in the series 0, 1, 2, 4, ..., 6400 $\mu\text{g./ml.}$ A 1 mm. loopful of a 24 hr. broth culture of the strain, usually derived from the slope stored in the refrigerator, was inoculated into 3 ml. of broth. A loopful of this dilution was then spread on a small area of each of the plates. It was possible to test at least twenty strains on one set of plates.

Stability of the strains. The stability during passage through streptomycin

free broth was investigated for a few strains of each of the types described. The strains were subcultured at daily intervals in broth, using a 3 mm. loopful as the inoculum. The streptomycin sensitivity of the strains was determined daily for the first few days, and later at approximately weekly intervals. A loopful of the highest dilution described under the method for viable counting was spread over a quarter of each of a series of plates containing a suitable range of streptomycin concentrations and the number of colonies counted. Cultures of known sensitivity were always included with each batch of strains tested in order to control the slight variations encountered with different batches of nutrient agar.

Table 1. Comparison of growth rates determined directly and by estimation from the indirect method

Strain	Growth rate, g_s or g_r	
	Estimated from g_I^*	Estimated directly
S	0.845	0.845
R 1	0.769	0.830
R 2	0.672	0.629
R 3	0.639	0.667
R 4	0.496	0.463

* Using equation (1) in the text, $x_1 = 3.00$.

g_s or g_r are the \log_{10} increases in numbers of sensitive or resistant organisms per hr. of growth in nutrient broth at 37°. These were obtained directly or by estimation from values of the fall in numbers of resistant organisms per day of subculture, g_I , as described in the text.

A note was made of the size and the amount of variation in the sizes of colonies on plain agar. On occasion the size of colonies was measured with a low-power microscope and a calibrated eyepiece.

Production of adapted strains. A series of tubes of broth containing a range of streptomycin concentrations was inoculated with two drops of a 24 hr. broth culture of the sensitive strain. After 24 hr. incubation another set was inoculated from the tube with the highest streptomycin concentration that showed growth. After five such transfers of two strains each showed partial growth in 1000 $\mu\text{g./ml.}$ These strains were tested for growth rate, streptomycin sensitivity and stability as described previously.

RESULTS

Growth rates

The results of two experiments are given in Table 2. The strains derived from resistant colonies could be divided into three types. Type I strains yielded very small colonies on plain nutrient agar and were rapidly eliminated from a mixture with the sensitive strain. Type II strains yielded slightly larger colonies and were eliminated rapidly but at a slower rate than type I strains. Type III strains yielded larger colonies of variable size. They were eliminated at variable rates from the mixtures, usually less rapidly than either type I or

type II strains. They could be divided, as will be explained later, into two subtypes, IIIa and IIIb, but this distinction was not made in Exp. 1.

It seemed clear from these two experiments that the strains derived from any one plate seeded with 1 ml. of broth culture were usually of one type and where these were of type III they had the same growth rates. Occasionally

Table 2. *The classification and growth rates of resistant strains obtained by inoculating streptomycin plates with 1 ml. volumes of broth cultures of the sensitive strain*

Exp. 1. Average number of organisms in each 1 ml. culture 1.69×10^9 .

Total number of plates receiving 1 ml. culture: 37.

A. Plates yielding resistant colonies

Plate no.	No. of dependent colonies	No. of resistant colonies	Classification of colonies and differential growth rates, g_1					
			Type I		Type II	Type III		
1	0	1	—	—	—	-0.655		
2	0	1	—	—	—	-0.565		
3	0	6	-2.150	-2.165	—	—		
			-2.155	-2.255	—	—		
			-2.200	-2.240	—	—		
4	0	3	—	—	-1.488	—		
			—	—	-1.488	—		
			—	—	-1.425	—		
5	0	2	—	—	—	-1.093	-0.983	—
6	1	2	-2.210	-2.195	—	—		
7*	0	21	—	—	—	—		
8 (seeded from part of a 50 ml. culture)	25	23	-2.195	-2.195	-1.638	-0.270	-0.295	—
			-2.160	-2.210	-1.518	-0.248	-0.263	—
			—	—	-1.448	-0.265	-0.278	—
			—	—	—	-0.560	-0.565	—
			—	—	—	-0.558	-0.580	—
			—	—	—	-0.575	-0.575	—
			—	—	—	-0.585	-0.713	—
			—	—	—	-0.623	-0.573	—
			—	—	—	—	—	—

B. Plates on which only dependent organisms grew: Number of plates: 14; numbers of dependent colonies on these plates: 2, 3, 4, 1, 5, 2, 2, 2, 1, 1, 1, 2, 1, 1.

C. Number of plates not yielding growth: 16.

* One plate yielded twenty-one type III colonies, whose growth rates were not tested.

two types were present, but the colonies could always be distinguished from each other on the original streptomycin plates. For this reason in the third and fourth experiments detailed in Table 3, where there were numerous colonies of the same size on a streptomycin plate only a few of them were sampled. Where more than one size of colony was present on any plate samples of each size were tested. In the fourth experiment 1.5 ml. of broth culture were inoculated on to plates containing 1000 instead of 100 $\mu\text{g./ml.}$ streptomycin and only two colonies from each plate were tested. The main purpose of this experiment was to obtain a number of separate clones of type III strains for an investigation of the variability of their growth rates. As will be noted later

Table 2 (cont.)

Exp. 2. Average number of organisms in each 1 ml. culture 2.28×10^9 .

Total number of plates receiving 1 ml. culture: 29.

A. Plates yielding resistant colonies: 11

Plate no.	No. of dependent colonies	No. of resistant colonies	Classification of colonies and differential growth rates, g_I			
			Type I	Type II	Type IIIa	Type IIIb
1	0	1	—	—	—1.390	—
2	2	2	—	—	—0.733 —0.743	—
3	2	7	—2.895	—	—0.545 —0.474 —0.505 —0.534 —0.540 —0.494	—
4	0	1	—2.265	—	—	—
5	1	5	—2.270	—	—0.617 —0.549 —0.533 —0.567	—
6	1	1	—	—1.430	—	—
7	0	2	—	—	—	—0.377 —0.303
8	3	4	—2.165 —2.295	—1.240 —1.475	—	—
9	0	3	—2.210	—	—0.526 —0.468	—
10	1	4	—	—	—0.498 —0.572 —0.556 —0.654	—
11	0	1	—	—	—0.632	—
12 (seeded from part of a 50 ml. culture)	8	17	—2.195	—	—0.240 —0.238 —0.271 —0.241 —0.256 —0.603 —0.537 —0.501 —0.537 —0.625 —0.608 —0.613 —0.588 —0.614 —0.646 —0.567	—

B. Plates on which only dependent organisms grew: number of plates: 7; numbers of dependent colonies on these plates: 1, 2, 1, 1, 2, 3, 4.

C. Number of plates not yielding growth: 11.

type II strains were inhibited by streptomycin at $1000 \mu\text{g./ml.}$ so that none of them was obtained on these plates.

Values of the differential growth rates obtained in these experiments were subjected to standard analysis of variance methods. The following conclusions could be drawn:

(1) Within the limits of experimental error there was no difference in the growth rates of strains belonging to the same clone.

(2) All type I strains had identical growth rates.

(3) All type II strains had identical growth rates which were more rapid than those of type I strains.

(4) Each clone of type III strains had its own characteristic growth rate which differed from those of other clones.

Table 3. *Classification and growth rates of samples of the resistant strains obtained by inoculating streptomycin plates with 1 ml. volumes of broth cultures of the sensitive strain*

Exp. 3. Average number of organisms in each 1 ml. culture 1.29×10^9 .

Total number of plates: 41.

A. Plates yielding resistant colonies: 8.

Plate no.	No. of dependent colonies	No. of resistant colonies	Classification of colonies and differential growth rates, g_I				
			Type I		Type II	Type IIIa	Type IIIb
1	0	8	-2.000	-2.070	—	—	—
			-2.165				
2	0	1	-2.055		—	—	—
3	5	1	—		—	-0.475	—
4	0	10	-1.995	-1.950	—	—	—
			-2.135	-2.050			
			-2.185	-2.175			
5	0	2	-2.095	-1.915	—	—	—
6	2	6	-2.325		-1.065	-1.135	—
					-1.275		—
7	0	1	—		—	-0.640	—
8	1	4	—		—	-0.648	-0.623
						-0.423	-0.385

B. Plates on which dependent organisms only grew: number of plates: 10; numbers of dependent colonies on these plates: 2, 1, 4, 2, 1, 2, 2, 6, 1, 2.

C. Number of plates not yielding growth: 23.

Exp. 4. Broth cultures of 1.5 ml. volume inoculated on to plates containing streptomycin 1000 $\mu\text{g./ml.}$

Plate no.	No. of colonies on plate	Classification of sample of two colonies from each plate and differential growth rates, g_I			
		Type I		Type IIIa	Type IIIb
1	26	-2.145	-2.275	—	—
2	5	-2.280	-2.395	—	—
3	59	—		-0.720	-0.648
4	2	-2.230	-2.250	—	—
5	5	—		-0.763	-0.810
6	5	—		-0.723	-0.828
7	6	—		—	-0.255
8	6	-2.075	-2.340	—	-0.300
9	136	—		—	-0.288
		-2.350		-0.673	-0.358
10	2	—		-0.923	—
11	1	—		—	—
12	9	-2.225*		—	—
13	2	—		—	-0.268
					-0.358

* The other colony of this pair was dependent.

The variability of the growth rates of type III strains was investigated by determining the differential growth rate, g_r , of twenty strains each derived from a separate clone. Strains were subcultured from the stock slopes into tubes of broth and the differential growth rates measured simultaneously. For comparison, fifteen well separated colonies of the parent sensitive strain were inoculated into tubes of broth. A mixture was made of each of these cultures with the same type III resistant strain whose growth rate was known, and the rate at which it was eliminated from the mixture of each of the sensitive strains was measured. Assuming a value of g_s of 0.845 for the sensitive strain (see Table 1) it was possible to calculate a series of values of g_r for the twenty resistant strains and of g_s for the fifteen sensitive strains from equation (1).

Table 4. *Comparison of growth rates of twenty type III resistant strains and fifteen sensitive strains*

Twenty type III strains grown with the sensitive strain

$g_s = 0.845$ (assumed). g_r determined for each strain $x_1 = 3.000$

From equation (1) $g_r = 0.845 \left(\frac{3 + g_r}{3} \right)$.

Average of values of $g_r = 0.7209$.

Standard deviation of $g_r = 0.0675$.

Fifteen sensitive strains grown with a single type III resistant strain

g_r for the resistant strain = -0.5942.

Assuming $g_s = 0.845$, then from equation (1) g_r for this strain = 2.0329. $x_1 = 3.000$.

For the fifteen sensitive strains from equation (1)

$$g_s = \frac{2.0329 \times 3}{3 + g_r}.$$

Average of values of $g_s = 0.8286$.

Standard deviation of $g_s = 0.0437$.

The results are given in Table 4. The standard deviations of the two series were compared using variance of variance = $2\sigma^2/(n-1)$, where σ is the variance. For this comparison $t = 1.61$ and $P = 0.2-0.1$. One can therefore conclude that type III strains, arising independently, show the same degree of variability of their growth rates as would strains derived from individual bacteria of the sensitive population.

Although resistant strains of one type, or occasionally two types, were obtained from 1 ml. broth cultures, the sample of the 50 ml. cultures in Exp. 1 (Table 1) yielded strains belonging to types I-III and the sample of the 50 ml. culture in Exp. 2 (Table 1) yielded types I and III. Furthermore, in both cases the type III strains could be divided into two groups each with a characteristic growth rate. These facts could be explained on the assumption that each clone was derived from a single mutant cell previous to the application of streptomycin. The chances of more than one mutant in 1 ml. volumes of culture would be slight, but would be 50 times higher in the 50 ml. cultures.

All the type III strains obtained in these four experiments grew at a slower

rate than the sensitive strain. However, it was possible, by selecting from a large volume of broth culture of the sensitive strain, to obtain a resistant strain which grew as rapidly as its parent.

Table 5. *The diameter of colonies of the sensitive and various resistant strains compared with their growth rates by the indirect method*

Strain	g_I	Average colony diameter (mm.)	Standard error	t	P
Type I	-2.275	0.526	0.0331	{ 1.68	0.1
Type I	-2.080	0.605			
Type II	-1.540	0.898		{ 1.28	0.3-0.2
Type II	-1.485	0.838			
Type IIIb	-0.255	2.011			
Type IIIa	-0.923	1.514		{ 2.61	0.02-0.01
Type IIIa	-1.390	1.183			
Sensitive	—	1.940			
Adapted 1	-1.765	0.395			
Adapted 2	-0.995	0.518			

The standard error of the average colony diameter was obtained from the intra-block error term of the analysis of variance.

Colony sizes

The colony sizes of two type I strains, two type II strains, two type IIIa strains, one type IIIb strain, the two adapted strains and the sensitive strain were measured simultaneously. A suitable dilution of each strain was spread on six quarters of nutrient agar plates without streptomycin. Differences between plates were eliminated by the use of the balanced incomplete blocks arrangement (Fisher & Yates, 1948). After incubation for 14 hr. at 30° and 8 hr. at 37° thirty well separated colonies of each strain were measured. The results are given in Table 5. No difference was found between colony size of the two type I or the type II strains, but the difference between colony sizes of the two adapted strains was significant. There was a reasonable correlation between growth rates and colony size.

Streptomycin resistance

Type I strains grew in the presence of 6400 $\mu\text{g./ml.}$, the highest concentration used. The number of colonies on these plates was not significantly different from the number on control plates without streptomycin. However, as the concentration of streptomycin in the plates increased growth became progressively slower, and at 3200 and 6400 $\mu\text{g./ml.}$ colonies were often not visible till they had been incubated for 48 hr. at 37°. All the strains were identical in their behaviour.

Type II strains also behaved identically. All strains were inhibited by *c.* 400 $\mu\text{g./ml.}$ There was no significant difference between the numbers of colonies growing on medium containing no streptomycin or 100 $\mu\text{g./ml.}$, and the colony size was only slightly smaller in streptomycin plates. The number of colonies growing on plates containing 200 $\mu\text{g./ml.}$ was less than on control plates and they were small and irregular in size.

Rather unexpectedly type III strains were found to behave in two quite distinct ways. Type IIIa strains grew well in the presence of 6400 $\mu\text{g./ml.}$ and the colonies were almost as large as on plates without streptomycin. There was no difference in the number of colonies on plates with or without streptomycin. Type IIIb strains grew well in 800 $\mu\text{g./ml.}$, but in 1600–6400 $\mu\text{g./ml.}$ growth was very slow and the colonies only became visible after 48 hr. incubation at 37°. This slowing of growth, occurring suddenly in concentrations over 800 $\mu\text{g./ml.}$, was characteristic of all these strains and clearly distinguished them from type IIIa strains. The number of colonies that developed at all streptomycin concentrations did not differ from the number on streptomycin free plates. Strains in Exp. 1 were not tested for resistance in a manner that would distinguish between types IIIa and IIIb, but in Exps. 2–4 (Tables 3 and 4) it can be seen that all the type III strains in each clone were either IIIa or IIIb.

Stability on subculture in broth

Type I strains

Daily subcultures of eight strains from four separate clones were carried out in broth. Each day an inoculum containing *c.* 100 organisms was spread on plates with and without streptomycin 100 $\mu\text{g./ml.}$ Large colonies began to appear on plates without streptomycin after one or two subcultures, and within 1–3 days they had replaced the original small colonies. Organisms from these large colonies were inhibited by a streptomycin concentration 2–4 times greater than that which would inhibit the sensitive strain. Two of the colonies were inoculated into tubes of broth and were then subcultured daily 31 times. At the end of this period the colonial morphology and streptomycin resistance were unchanged. The growth rate, by the direct method, of one of these strains was 0.632, only slightly less than 0.845 for the sensitive strain. The distinction between the original type I colonies and the large colonized variants derived from them was well marked and there were no intermediate forms.

Table 6. *The average diameter of ten colonies from resistant strains grown on streptomycin free agar before and after forty-two successive daily subcultures in broth*

Strain	Average diameter of colonies and its standard error	
	Before subculture (mm.)	After subculture (mm.)
Type II	0.90 \pm 0.014	1.73 \pm 0.044
Type IIIa	1.05 \pm 0.025	1.46 \pm 0.034
Type IIIb	1.56 \pm 0.034	1.70 \pm 0.044
Sensitive	1.80 \pm 0.048	

Type II strains

These strains were also unstable though in a less clear-cut fashion. There was a slight fall in resistance occurring after 5–16 daily subcultures. The strains were now inhibited by a streptomycin concentration of 100 $\mu\text{g./ml.}$

At the same time larger colonies of two sizes began to appear on streptomycin free plates. Usually, though not invariably, the larger colonies were less resistant than the small ones. After a total period of subculture of 2-6 weeks a further fall in streptomycin resistance occurred. The strains now contained a majority of organisms only 4-8 times less sensitive than the sensitive strain, but there was also present a small proportion of the more resistant organisms inhibited by 100 $\mu\text{g./ml.}$ During further subculture this proportion varied from 1 to 50 % of the total culture, but showed no signs of being eliminated. By this time all colonies on streptomycin free plates were of fairly uniform size and as large as the parent strain. Measurements of colony size are given in Table 6. These results appear to indicate that the fall in resistance occurred in two stages, although complete reversion to the original level of sensitivity of the parent strain did not occur.

Type III strains

A group of three type IIIa strains with widely different growth rates and a type IIIb strain were still able to grow in the presence of 6400 $\mu\text{g./ml.}$ streptomycin after forty-two daily subcultures in broth. The type IIIb strain also showed the same slow growth in streptomycin concentrations of 1600-6400 $\mu\text{g./ml.}$ as it did originally.

Type III strains with a slow growth rate and a small colony size, when subcultured in broth and plated out at intervals on plates without streptomycin, produced after a few days a number of colony variants which were larger than the original colonies. After about a month's subculture, as is shown in Table 6, the colonies were almost as large as those of the sensitive strain. This phenomenon was investigated in greater detail in an experiment in which two clones of type III resistant strains, one with growth rates almost as rapid as the sensitive strain and the other with slow growth rates, were mixed with the sensitive strain and the fall in the numbers of resistant organisms determined. This was carried out immediately after isolation and after subculture of the resistant strains for 7 and 21 days in streptomycin free broth.

The slowly growing strains increased their growth rates after subculture in plain broth, but to differing extents. Also, during the periods of the tests done after 7 and 21 days' subculture, the rate for any one strain was more variable. In particular, the behaviour of one strain in the mixture made after 7 days was notable in that for 8 days it had a slow growth rate; then it grew as well as the sensitive strain for 5 days and finally the growth rate again became slow. Then again, one of the two strains from the clone that grew rapidly maintained its growth rate in the mixture made after 7 days, but the other strain, after maintaining its growth rate for 6 days, began to grow increasingly slowly. In the mixture made after 21 days the same phenomenon occurred but the behaviour of the strains was reversed.

One could conclude that the constancy of the growth rate characteristic of type III resistant strains belonging to a single clone was lost after 21 days' subculture in streptomycin free medium and was replaced by a variability similar in degree to that found between strains derived from a number of

different clones. Although most strains increased their growth rates a few spontaneously decreased their growth rates for shorter or longer periods.

Type IIIa strains tested shortly after isolation produced colonies of about the same size whatever the concentration of streptomycin in the plates. However, after forty-two daily subcultures in broth, the three strains tested yielded colonies that were smaller with progressively increasing streptomycin concentrations, so that on 400 $\mu\text{g./ml.}$ or above the colonies were smaller than those produced by the original strain.

Growth rates of resistant strains mixed in varied proportions with the sensitive strain. It might be thought that the behaviour of mixtures of equal quantities of sensitive and resistant organisms would not reflect the population dynamics in other cases where the proportions differed widely. When two type III strains were mixed in widely different proportions with the sensitive strain their growth rates were found to be the same whatever the original proportion of the resistant to sensitive organisms. The proportions of resistant to sensitive organisms in this experiment were 1:10³, 1:1, 10³:1 and 10⁶:1 (in the last case the inoculum contained about fifty sensitive organisms). In the cases where the resistant organisms predominated the differential growth rates were calculated by an extension of the method used for obtaining equation (1).

Adapted strains

The results of testing the differential growth rate and the streptomycin sensitivity of the two adapted strains are shown in Table 7. The average colony sizes on nutrient agar, compared with those of other resistant types, are

Table 7. *Comparison of the growth rates and streptomycin sensitivities of the adapted strains with type I and type II strains*

Strain		Differential growth rate (g_1)	Minimal inhibitory concentration of streptomycin ($\mu\text{g./ml.}$)
Type	No.		
Adapted	1	-1.765	1600
Adapted	2	-0.995	1600
I	3	-2.080	> 6400
I	4	-2.275	> 6400
II	5	-1.485	400
II	6	-1.540	400

shown in Table 5. The two strains had different growth rates, both being rather slow and corresponding most closely to those of type II resistant strains. They were both partially resistant to streptomycin, being inhibited by 1600 $\mu\text{g./ml.}$ After 2 days' subculture in broth without streptomycin there was no change in streptomycin resistance. After 3 days' subculture, however, large colonized variants began to appear which were less resistant to streptomycin. After 7 days' subculture both strains were mainly composed of organisms inhibited by 8-32 $\mu\text{g./ml.}$, together with about 10% of organisms inhibited by 200 $\mu\text{g./ml.}$ Adapted strains corresponded in their behaviour on

subculture to type II strains except that the changes occurred more rapidly. They also correspond in streptomycin sensitivity and growth rates most closely to type II strains, although in each case there were definite differences in behaviour.

Mutation rate

The mutation rates to resistance of types I–III, to resistance of all types and to dependence were calculated using Newcombe's (1948) formula (1):

$$\text{Mutation rate} = -\frac{(\log_e 2)(\log_e P_0)}{N},$$

where P_0 = proportion of cultures without mutants and N = number of bacteria per culture. This estimation is unaffected by the relative growth rates of sensitive and resistant strains.

The results from Exps. 1–3 are given in Table 8. Separate rates for types III*a* and III*b* could not be calculated because the data were insufficient. However, out of nineteen clones tested in Exps. 2–4, fifteen were type III*a* and four type III*b*. The mutation rates to the different resistant types do not differ significantly from each other.

Table 8. *Mutation rates for resistant and dependent strains*

	Experiment			Average
	1	2	3	
	Mutation rate $\times 10^{-11}$			
All resistant types	8.6	14.5	11.7	11.6
Resistant type I	2.2	5.8	7.0	5.0
Resistant type II	0.87	2.2	1.4	1.5
Resistant type III	4.7	9.8	4.0	6.2
Dependent	21.3	18.1	20.5	20.0

DISCUSSION

These experiments show that streptomycin-resistant strains obtained from a sensitive population by single step selection could be divided into three main types. Type I strains had a slow growth rate, were completely resistant, though growing poorly at high streptomycin concentrations, and were markedly unstable when subcultured in broth. Type II strains had a slightly more rapid growth rate, were partially resistant and were unstable when subcultured in broth, though much less so than type I strains. Type III strains had a variable growth rate, usually more rapid than type I or type II strains, were completely resistant and were stable on subculture. Type III strains could be subdivided on the basis of only one character. Type III*a* strains grew almost as well in the absence as in the presence of high concentrations of streptomycin, whereas type III*b* strains grew much more slowly on plates containing 1600 $\mu\text{g./ml.}$ or over. These characters were all quite clearly defined and in no case was there any doubt as to which type any strain should be assigned.

Evidence for the existence of streptomycin-resistant mutants in a sensitive population before the selective contact with the drug has been presented by

Alexander & Leidy (1947), Demerec (1948), Meads & Haslam (1949), Scott (1949), English & McCoy (1951) and Lederberg & Lederberg (1952).

These results provide further evidence for this view, since the only reasonable explanation for the occurrence of clones of resistant colonies having identical characters is that they were each derived from a single mutant cell which had the opportunity to divide a few times before contact with streptomycin.

The 1 ml. broth cultures of the sensitive strain were inoculated with a number of organisms too small to contain any resistant mutants. Since strains of each of the types described were obtained from a number of these independent cultures it is reasonable to consider that each type arose by a separate mutation. If one assumes that mutation rate to types I-III resistance are the same, one can calculate from the data in Tables 2, 3 and 8 how many 1 ml. cultures in Exps. 1-3 would be likely to contain mutants of two different types. These calculated values are 0.1, 0.9 and 0.6, whereas in these experiments respectively 0, 4 and 1 cultures actually contained mutants of two types. Using the same data one can calculate that the most likely number of resistant clones to be obtained from the 50 ml. volumes of the sensitive strain in Exps. 1 and 2 would have been about 3 and 9. In fact, the numbers found were 4 and 3. It is therefore reasonable to assume that the mutations arose independently of each other.

None of the resistant strains which was subcultured in streptomycin free broth reverted to the sensitivity of the sensitive strain. All type I strains and the large colonied variant strains derived from them had slower growth rates than the sensitive strain. Type II strains had slower growth rates for at least the first fortnight of daily subcultures. Type III strains usually had slower growth rates, and even where these were as rapid as the sensitive strain the data in Fig. 4 indicate that they tended to revert permanently or temporarily to a slower rate. A mixed population, containing initially only fifty sensitive organisms and a much larger number of a resistant strain with a slower growth rate, was shown after 3 days' subculture to contain a majority of sensitive organisms. It may be concluded, therefore, that back mutation to a strain identical with the sensitive strain does not occur or is extremely rare. This assumes that the back mutant, if it occurred at all, would appear sufficiently early in the growth in one tube for its progeny to have a chance of being transmitted in the inoculum to the next tube. Such a condition is likely to have been fulfilled in one of the large number of tubes in which the resistant strains were subcultured.

In two experiments with large volumes of broth cultures of the sensitive strain the proportion of resistant and dependent organisms to sensitive organisms was found to be $1:5.8 \times 10^8$ and $1:8.2 \times 10^8$. The maintenance of this ratio between a large number of sensitive organisms and a small number of resistant ones can be explained as a balance between two tendencies. The population of resistant organisms is constantly increased by new mutants. If the resulting resistant strains grew as well as the sensitive strain they would eventually replace it, since it appears that back mutation either does not occur

or occurs very rarely. However, this tendency for the resistant population to increase is counterbalanced by the slower growth rate of resistant strains and the failure of dependent strains to grow for more than two divisions (Newcombe & Hawirko, 1949). As an example in which this does not occur English & McCoy (1951) found that streptomycin-resistant variants of *Staphylococcus aureus* grew better than the sensitive strain in a semi-synthetic medium and were able to obtain a streptomycin-resistant strain by inoculating a large number of tubes of this medium with sensitive organisms.

Speculations about the nature of resistance to streptomycin tend to emphasize how little is known about it. Two comments can, however, be based on these results. The variability of sensitive strains derived from separate colonies suggests that individual bacilli in a sensitive population have characteristic growth rates which are transmissible to daughter cells during several periods of daily subculture. The variability in these growth rates was also found in type III resistant strains and is most likely to be due to several factors. Their effect has, however, been completely suppressed in types I and II strains, whose growth rates appeared identical. An investigation into the causes of variability among sensitive and type III resistant strains might provide evidence as to the nature of the mechanism of types I and II resistance.

The second comment springs from consideration of the colony sizes of resistant strains in the absence and in the presence of streptomycin. Oginsky, Smith & Umbreit (1949) have suggested that streptomycin acts by preventing an oxaloacetate-pyruvate condensation and that this condensation does not occur in resistant strains. From this hypothesis one would expect the growth rate of resistant strains to be unaffected by streptomycin. However, type I colonies were found to be very much smaller in the presence of high streptomycin concentration than in its absence. Type II strains were inhibited at concentrations over 400 $\mu\text{g./ml.}$ Type III*b* colonies were small in the presence of 1600 $\mu\text{g./ml.}$ or more. Type III*a* colonies, although initially of equal size in the presence or absence of streptomycin, were much smaller in its presence after a period of subculture in broth. Any hypothesis, such as this, that explained the action of streptomycin by an effect on a site which is present in sensitive strains but completely absent in resistant strains is thus unlikely to provide a full explanation.

Newcombe & Nyholm (1950) have provided evidence from experiments with sexual matings of *Bact. coli*, strain K-12, that streptomycin resistance and dependence arise through mutation in the same single gene locus. It would appear likely that their resistant strains were type III since, under the conditions they describe for obtaining their strains, type II colonies would not appear and type I colonies would be very small. Whether types III*a* and III*b* strains arise from the same or a different locus is undetermined.

Type II strains resemble the adapted strains most closely. There is evidence that several mutational steps are involved in the production of adapted strains (Demerec, 1948), and that some of these involve the locus for resistance and dependence (Newcombe & McGregor, 1951). The stepwise fall in resistance of

type II strains when subcultured in the absence of streptomycin suggests that multiple mutations are also involved in the production of these strains. Since they were obtained by a single step selection, these mutations must have been present simultaneously in the same organism and each must therefore be relatively frequent in occurrence.

Type I strains subcultured in the absence of streptomycin produced stable variant strains with a higher growth rate and lower resistance. This would suggest either that two mutations are involved in the production of these strains, from one of which there are frequent back mutations, or that the variant strains result from the effect of a blocking gene on an original single mutation.

The genetic mechanisms need further investigation, and the occurrence of these types of streptomycin resistance must be confirmed in other strains of *Bact. coli* before generalizations can be made.

This work was carried out during the receipt of a grant from the Medical Research Council, and with the technical assistance of Mr D. J. Rose. I am indebted to Dr W. Hayes for his interest and criticisms.

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(Received 5 August 1952)

Penicillinase Adaptation and Fixation of Penicillin Sulphur by *Bacillus cereus* Spores

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With a Note by C. J. PERRET on the Biosynthesis and Isolation of ^{35}S -labelled Benzylpenicillin

SUMMARY: In contrast to vegetative cells, *Bacillus cereus* spores do not react to treatment with penicillin by increased production of penicillinase when subsequently grown in a penicillin-free medium. Neither do spores show specific fixation of penicillin sulphur, with maximum absorption at 1.0 unit/ml., which is characteristic of vegetative cells. However, treatment of vegetative cells with penicillin shortly before or during sporulation leads to the formation of 'adapted' spores able to form penicillinase up to 10 times more rapidly than untreated spores when subsequently germinated and grown in a penicillin-free medium; and specific uptake of penicillin S on spore material occurs after mechanical disruption of the spore-wall. It is concluded that the fully mature and undamaged spore-casing is impermeable to penicillin.

Suspensions of vegetative cells of *Bacillus cereus* respond specifically to treatment with penicillin by rapidly producing penicillinase when subsequently grown in a penicillin-free medium (Pollock, 1950). The rate of penicillinase-formation is determined by the concentration of penicillin used, with a maximum at about 1.0 unit/ml. ($1.7 \times 10^{-6}\text{ M}$), and is proportional to the specific uptake of penicillin sulphur by the cells—as followed by the use of ^{35}S -labelled penicillin (Pollock & Perret, 1951). Suspensions of spores, however, show no such response to penicillin when treated under similar conditions. Their rate of penicillinase formation is identical with the 'basal' rate shown by untreated spores; and their fixation of penicillin S is of the 'non-specific' type, without the disproportionately high uptake at concentrations below 1 unit/ml., characteristic of vegetative cells. The work reported here was undertaken in an attempt to find an explanation of these differences.

METHODS

Medium. 10 g. Evans peptone, 3 g. Lemco and 2 g. of NaCl were dissolved in 1 l. of water. The pH value was adjusted to 7.6 and the medium steamed for 30 min. and filtered through paper to clarify. The pH value was then readjusted to 7.0 and the medium autoclaved at 10 lb. for 20 min. 'Sporulation' (S) broth prepared in this way will allow 95 % of *B. cereus* cells to undergo sporulation after 16 hr. growth from a suitable inoculum. If, however, the so-called 'phosphates' are removed—for instance by adjusting the pH value too high before steaming, and filtering off the resultant precipitate—sporulation will be almost completely prevented.

Inoculum. One drop of various dilutions of a standard suspension of *B. cereus* NRRL 569 spores, having a viable count of 3×10^8 /ml., into 50 or 100 ml. of medium.

Vegetative cell suspensions. A 16 hr. culture incubated on a shaker at 35°, after inoculation with one drop of 10^{-6} dilution of standard spore suspension into 100 ml. of medium, was centrifuged, the cells washed once and made up to standard opacity by reference to an opacity/dry weight curve.

Spore suspensions. Cultures similar to that used for preparation of vegetative cells were allowed to remain shaking at 35° for 40 hr., by which time most of the vegetative cell material had lysed. The spores were then centrifuged and washed once. They were resuspended in water and heated at 60° for 1 hr., centrifuged down again, washed three times and finally resuspended to standard opacity. This gave a fairly homogeneous suspension of clean spores more or less free from vegetative cell debris.

Penicillinase production was followed aerobically at 35° in *S* medium containing 1 % (w/v) gelatin, samples being removed into 0.00084M-oxine and assayed manometrically, all details being exactly as previously described (Pollock, 1950). Results are expressed as μ l. CO₂ released (by the penicilloic acid) from a bicarbonate buffer at pH 7.0 and 30° by 1 ml. in 1 hr.

Radioactive benzylpenicillin labelled with ³⁵S having an initial specific activity of about 0.18 μ c./unit was prepared by C. J. Perret as described in the Appendix, and kept sealed in ampoules in N₂ after freeze-drying. The penicillin was dissolved in 0.01M buffer at pH 7.0, and its concentration determined by bio-assay, using two dilutions, each in octuplicate, against similar dilutions of a standard solution (cup-plate technique, giving an accuracy of ± 5 %). Analysis by paper strip chromatography showed that 90 % of the ³⁵S was in the penicillin molecule, and that most of the rest was in penicilloic acid probably formed after the penicillin solution had been added to the paper. Apart from penicilloic acid, non-penicillin S impurity in the material used for these experiments was almost certainly considerably less than 5 %.

The solution of benzylpenicillin was kept frozen at -10°, and, if needed again more than 7 days later, was re-assayed immediately before use. Loss of activity at -10° was very slight and did not amount to more than 10 % in 7 days. Radioactive penicilloic acid was prepared enzymically by incubating equal volumes of a solution of radio-penicillin at 100 units/ml. and cell-free penicillinase (activity: 5000 μ l. CO₂/ml./hr.) for 60 min. at 35° immediately before use.

Assay of penicillin sulphur fixed on the cells after radio-penicillin treatment for 1 hr. at 0°, washing and freeze-drying on polythene disks, was done on a Geiger counter using the technique described by Pollock & Perret (1951). The only modification found necessary was the addition of one standard drop of 3 % gelatin to the spore suspension on the disk, followed by thorough mixing before freezing in order to prevent the fine dust of spores scattering off the disk while drying. This amounted to the addition of 0.66 mg. of gelatin to each disk and was allowed for in the final calculation. Vegetative

cells became sufficiently glutinous on freeze-drying not to need such treatment.

Disintegration of cells. (a) Vegetative cells were disrupted by 10 min. shaking with an approximately equal volume of glass ballotini on a Mickle vibrator, using a small quantity of Silicone DC Antifoam A. This caused almost complete disintegration of the cells.

(b) Spores were refractory to disruption on the Mickle vibrator or by treatment with ultra-sound. Fairly satisfactory, though not complete, breaking (see Pl. 1, fig. 7) was eventually achieved by three successive crushings at -35° , -55° and -22° with intervening thaws at $+2^{\circ}$ in the Hughes press (Hughes, 1951).

RESULTS

Preliminary

When suspensions of spores were treated with penicillin for 1 hr. at 0° and subsequently washed free of penicillin and incubated (i.e. germinated and grown) for 2–3 hr. aerobically in *S* broth containing 1% gelatin, no more penicillinase was formed than with untreated spores. This failure to adapt to pretreatment occurred whatever concentration of penicillin was used; and is

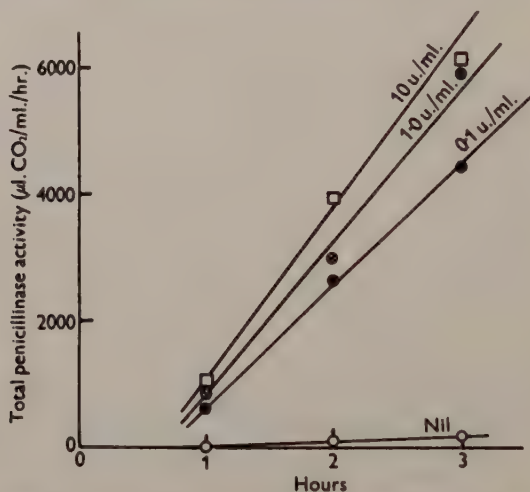


Fig. 1. Penicillinase production by germinating *B. cereus* spores (2 mg./ml.) incubated aerobically at 35° in 1% gelatin-broth containing 0, 0.1, 1.0 and 10 units penicillin/ml.

in marked contrast to the behaviour of vegetative cells which react to such treatment by subsequently forming penicillinase at a rate up to 100 times that of untreated cells. However, when spores are incubated in the gelatin-broth medium in the presence of varying concentrations of penicillin, the cells adapt rapidly and, indeed, behave like vegetative cells do under such conditions (Fig. 1). There is thus nothing associated with the early stages of germination and growth which prevents cells showing the phenomenon of penicillinase adaptation.

Moreover, when spores are pretreated with radioactive ^{35}S -labelled penicillin their S uptake is directly proportional to the concentration of penicillin used; and there is no disproportionately high uptake at low penicillin concentration as shown by vegetative cells (see Fig. 2). There is not, in fact, as with vegetative cells, any S uptake corresponding to the so-called 'specific' fixation of penicillin S suggesting a reaction between penicillin and some specific cell receptor which is saturated at a concentration of about 1.0 unit/ml. (see Pollock & Perret, 1951).

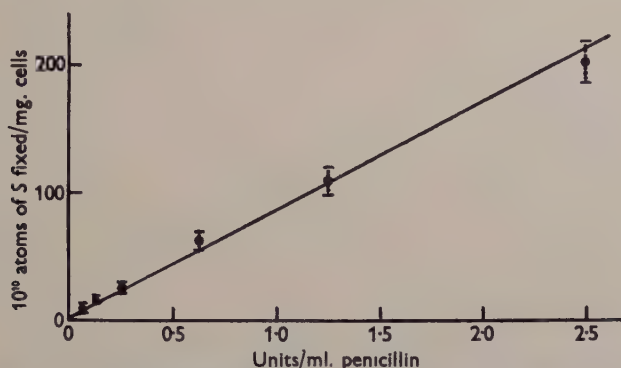


Fig. 2. Fixation of S on *B. cereus* spores following treatment at 0° for 1 hr. with different concentrations of ^{35}S -labelled penicillin, and subsequent washing. Limits of counting error shown for $P=0.05$.

There are two possible explanations for this difference: either (1) spores do not possess a specific receptor for penicillin S, or (2) spores are impermeable to penicillin. The latter alternative could, perhaps, be described more accurately as the hypothesis of there being some sort of a barrier preventing access of penicillin to the receptor—without any preconception of what sort of barrier it might be or where it functions.

Proauction of 'adapted' spores

If the impermeability hypothesis is correct, clearly it might be possible to produce adapted spores by allowing the penicillin to penetrate the cell and reach the receptor before the spores were fully mature. Then, if in fact some of the receptor substance were incorporated within the spore as they were being formed, such spores might be expected to form penicillinase at an increased rate when subsequently allowed to germinate and grow in a penicillin-free medium. This was indeed found to be the case. Fig. 3 shows the result of an experiment in which cells were treated in four different ways as follows:

Culture	Time of inoculation	Subsequent treatment
A	10 a.m. }	Addition of 100 units penicillin/ml. every 60 min. for 8 hr. on the following day from 10 a.m. to 6 p.m.
B	6 p.m. }	
C	10 a.m. }	
D	6 p.m. }	
		Nil

Four 100 ml. lots of medium were inoculated with one drop of a 10^{-6} dilution of standard spore suspension, two flasks at 10.0 a.m. and two at 6.0 p.m. and then incubated on the shaker at 35° . The onset of sporulation occurs consistently 16–18 hr. after adding an inoculum of this size, and, once begun, is complete (i.e. at least 95 % of cells have produced spores) in 7–8 hr. Thus at 10 a.m. the following morning sporulation was complete in *A* and *C* and just about to begin in *B* and *D*. The whole process of sporulation is

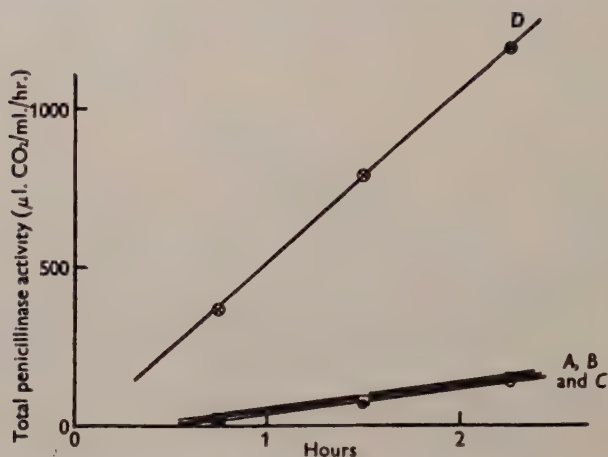


Fig. 3. Penicillinase production by germinating *B. cereus* spores 'primed' by penicillin-treatment during sporulation (*D*), compared with spores treated with penicillin after sporulation (*C*) and with untreated spores (*A* and *B*), see text. Medium: 1 % gelatin-broth. Aerobic incubation at 35° .

illustrated in Pl. 1, figs. 1–6. The continuous 8 hr. penicillin treatment was thus applied to fully formed spores in *C* and to cells during sporulation in *D*; *A* and *B* acting as untreated controls. Films of *B* and *D*, which were made during this period, showed no detectable difference in the speeds and extent of sporulation, confirming that the repeated addition of penicillin had no effect on the process. All cultures were then left shaking at 35° for a further 16 hr. (i.e. until 10.0 a.m. on the 3rd day) to allow lysis of remains of vegetative cells. Spore suspensions were then prepared, from each culture separately, by heating at 60° and thorough washing as described; and rates of penicillinase formation in 1 % gelatin-*S* broth followed. Fig. 3 shows that in *D* (cells treated with penicillin during sporulation) there was an approximately tenfold increase in the rate of enzyme formation; while *C* showed no significant difference from the untreated *A* and *B*. 'Adapted' spores could also be prepared from cultures treated with a single dose of 100 units penicillin/ml. just before the onset of sporulation.

It should also be pointed out that: (a) penicillinase activity was not detectable in any of the four spore suspensions before germination and growth in the broth; the term 'adapted' when applied to such cells is therefore perhaps misleading and might best be substituted by 'penicillin-primed';

(b) there was no significant change in the opacities of any of the four spore suspensions during germination and growth in broth for the $2\frac{1}{2}$ hr. the experiment lasted. Observations by phase-contrast illumination showed, however, that most of the cells had germinated and were beginning to divide. When incubation was prolonged, increase in opacities occurred, but to the same extent in all cultures. There were no observed differences between the suspensions apart from their ability to form penicillinase.

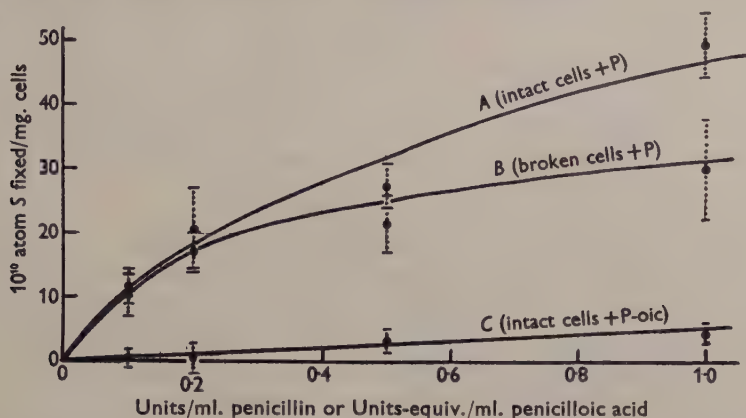


Fig. 4. Fixation of S on *B. cereus* vegetative cells after treatment for 1 hr. at 0° with different concentrations of ³⁵S-labelled penicillin or penicilloic acid followed by washing. A, intact cells; B, broken cells; C, intact cells after similar treatment with penicilloic acid prepared by enzymic hydrolysis of ³⁵S-labelled penicillin. Limits of counting error shown for $P=0.05$.

Penicillin sulphur fixation by crushed spores

It was thought possible that if the inability of spores to fix penicillin S specifically at low concentration were due only to an impenetrable barrier in the spore wall, then it might be possible to demonstrate such fixation after disrupting the spores mechanically. This should be feasible as long as the penicillin-binding receptor substance was not completely soluble and thus not removed during washing of the broken spores after pretreatment with penicillin. Few, Cooper & Rowley (1952) reported that, with penicillin-treated staphylococci, the penicillin was fixed largely on that portion of the cell which remained as the insoluble centrifugable deposit after cell disintegration. A preliminary experiment with *B. cereus* in which the solid debris after disruption of vegetative cells was treated with low concentrations of radioactive penicillin by the usual technique, showed the penicillin S to be specifically fixed by this material to about the same extent as by intact cells (Fig. 4). It seemed therefore reasonable to assume that mechanical disintegration of cells did not chemically or physically alter the properties of the cell material enough to cause any change in the ability to fix the penicillin S.

A suspension of spores was then crushed in the Hughes press. It differed from an intact spore suspension in its tendency to clump and was difficult to homogenize. When examined under the microscope a large proportion of

spores (30–40 %) appeared unaltered and were clumped together with other cells of ill-defined outline and with amorphous solid debris. Pl. 1, fig. 7, shows the appearance of the spore suspension after crushing. Although many intact cells remain, a number of empty burst spore cases and amorphous granular material are easily visible. The crushed spores were kept at 0° or below until just before use. They were then standardized to a concentration of 20 mg./ml. by weighing samples, and treated at a concentration of 2 mg./ml. with varying concentrations of ^{35}S -labelled penicillin for 1 hr. at 0° in parallel with similar

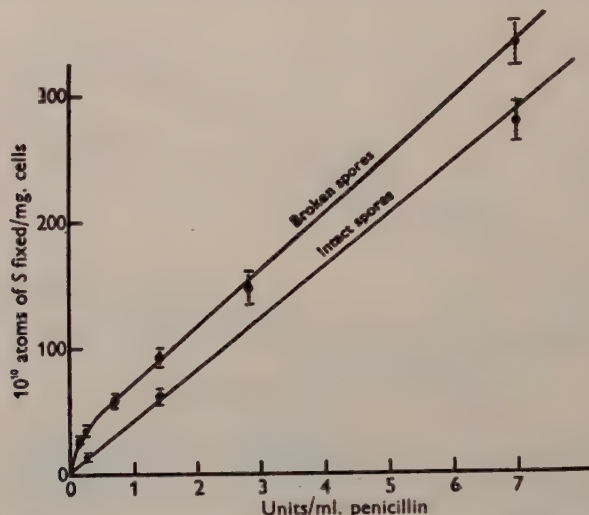


Fig. 5. Comparison of S fixation on intact and broken *B. cereus* spores after treatment for 1 hr. at 0° with different concentrations of ^{35}S -labelled penicillin, and subsequent washing. Limits of counting error shown for $P=0.05$.

concentrations of intact spores from the same original batch. After three washings, during which a considerable fraction of soluble material was removed from the crushed spores in the supernatant fluid after centrifugation, the amount of S fixed was estimated (Fig. 5). Although there is no great difference between the two preparations in the total S fixed at the higher levels of penicillin treatment, the divergence below a concentration of 1.0 unit/ml. is marked. While the fixation by intact spores is more or less proportional to the penicillin concentration over the whole range tested, there is a disproportionately high uptake by crushed spores at the lower penicillin concentrations and, apart from a higher 'non-specific' fixation, the curve is very similar to that shown by vegetative cells.

DISCUSSION

It seems reasonable to conclude from these experiments that spores contain a receptor for specific fixation of penicillin S and that their inability to react to treatment with penicillin—either in the form of adaptation to penicillinase production or by specific fixation of penicillin S—is due to the impermeability of the spore wall to penicillin. The mechanism of sporulation and the fact that

'penicillin-primed' spores can be formed after penicillin-treatment of vegetative cells also suggest that the specific induction of penicillinase formation occurs *inside* the vegetative cell. Pl. 1, figs. 1-6, show how the spore appears to be formed by an inclusion of part of the vegetative cell not involving the cell wall. Knaysi (1946) has clearly shown that endospore formation in *B. cereus* occurs entirely within the cell wall; and in the case described here it is not easy to see how material on the outside of the cell could be incorporated into the spore. Previous work (Pollock & Perret, 1951) has shown fairly conclusively that the receptor which specifically fixes penicillin S is the same as that which reacts to penicillin treatment by promoting penicillinase production. It is obviously simpler to assume that this receptor-substance is within the cell and is incorporated into the spore during sporulation, than to postulate some transmission of specific inducing power from this substance (if situated on the cell surface) through the cell wall and into the cytoplasm.

Reasons have previously been given (Pollock & Perret, 1951) for believing that the curve of penicillin S fixation shown by vegetative cells represents the combined effect of two different types of reaction: (a) 'specific' fixation of S on a specific cell receptor which is saturated at about 1 unit/ml. plus (b) 'non-specific' fixation on some cell material which adsorbs the S to an extent strictly proportional to the concentration of penicillin employed—at least up to 14 units/ml. This interpretation of two separate types of penicillin S fixation is supported by the present results which show that with spores the non-specific type of fixation can be transformed into the non-specific + specific type simply by rupturing the cell wall.

It seems possible that 'non-specific' fixation of penicillin S shown by spores represents that proportion which is absorbed on the outside of the cells. If so, it is likely that the same would apply to the non-specific fraction of S fixation from penicillin by vegetative cells. The higher value shown by spores (5 to 8×10^{11} atoms S/mg./unit penicillin/ml.—see Figs. 2 and 5) compared with that of vegetative cells (2.5 to 3.0×10^{11} atoms S/mg./unit penicillin/ml.—see Pollock & Perret, 1951) is in keeping with the much greater total surface area/weight ratio of spore suspensions. The only alternative interpretation, in fact, is that it does not represent fixation of S from penicillin at all, but from some non-penicillin S impurity present in the penicillin solution. An S impurity of less than 0.2% would be enough to account for the amount so absorbed. This, however, is rather unlikely, because in the case of vegetative cells the amount of S fixed from an enzymically produced penicilloic acid preparation (which would contain the same S impurities as the penicillin solution from which it was formed) was found to be only 20-25% (see Fig. 4) of the average amount of 'non-specific' S absorbed from penicillin. This hypothesis has little bearing on the problem of spore adaptation, but if true would be a simple explanation of the rather complex curve of penicillin S fixation by vegetative cells. This latter could then be regarded as being composed of a specific fraction absorbed by the specific receptor within the cell plus a non-specific fraction absorbed purely on the cell surface.

In this connexion it should be mentioned, in parenthesis, that much greater and very variable S fixation was found in cells treated with penicilloic acid formed from penicillin by alkaline hydrolysis as compared with enzymically produced penicilloic acid. The results reported previously (Pollock & Perret, 1951) regarding fixation of penicilloic acid S are therefore inaccurate and misleading since there is little doubt that most of what was regarded as penicilloic acid S was a S impurity produced, although in very small quantities, by the alkaline treatment of penicillin. Enzymic hydrolysis of penicillin is specific and less drastic than alkaline hydrolysis, and therefore much less likely to cause increase in impurity. However, even with the 'enzymic' preparation it is quite possible that the S fixed is not derived from penicilloic acid itself but represents some trace of impurity present in the original penicillin solution. In any case, the low amount of S fixed from penicilloic acid serves only to emphasize the highly specific uptake from penicillin at low concentrations.

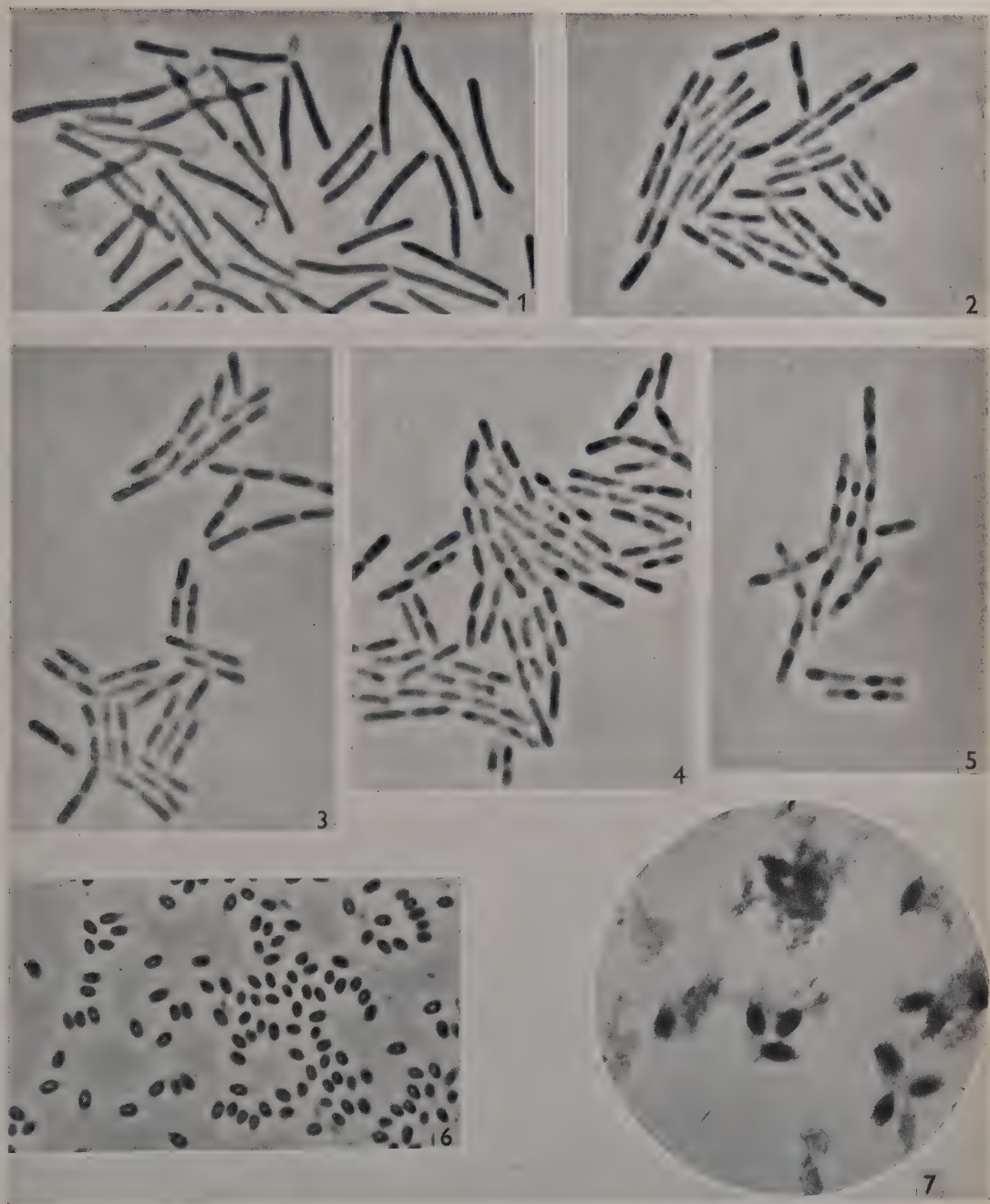
I am much indebted to Dr D. E. Hughes for undertaking the crushing of *B. cereus* spores in his press and for the electron micrograph of crushed spores (Pl. 1, fig. 7). I also wish to thank Mr M. R. Young for taking the photographs of the stages of sporulation, and Miss Daphne Towler for technical assistance.

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EXPLANATION OF PLATE

- Figs. 1-6. Sporulation in *B. cereus* during aerobic incubation at 35° in 'S' broth after inoculation with 1 drop of 10⁻⁸ dilution of standard spore suspension (see Methods). Phase contrast illumination. ×1440.
Fig. 1. 3 hr. Cells in 'log-phase' showing remains of spore-casings (from spore inoculum).
Fig. 2. 16 hr. Intracellular inclusion bodies (not present in cells during the log-phase) evenly distributed.
Fig. 3. 19 hr. Polarization of inclusion bodies to one end of cell. Some 'forespores' visible.
Fig. 4. 20 hr. Numerous 'forespores' and a few nearly mature spores.
Fig. 5. 23 hr. Mature spores present in most cells.
Fig. 6. 40 hr. Free spores after heating at 60° for 1 hr. and washing.
Fig. 7. Electron micrograph of spores after crushing in the Hughes press; showing empty spore cases, amorphous debris and some apparently intact spores. ×4000.



M. R. POLLOCK—PENICILLINASE ADAPTATION IN SPORES. PLATE 1

NOTE ON THE BIOSYNTHESIS AND ISOLATION OF
³⁵S-LABELLED BENZYL PENICILLIN

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This appendix describes in the form of laboratory notes a simple and rapid method for producing and isolating ³⁵S-labelled benzyl-penicillin. The process yields 5–15 mg. of the compound virtually free from radioactive contaminants and with a specific activity up to *c.* 0.2 μ c./unit. Approximately 20 % of the 'carrier-free' ³⁵S added to the medium is converted into penicillin. This process was evolved as the result of work ancillary to other researches and is not claimed to be original. But as there appears to be no published account which describes in detail the preparation of ³⁵S-penicillin, the information given here may be useful to other workers in the field.

Organism and maintenance of the culture

Organism. *Penicillium chrysogenum* WIS 48–701. Under suitable conditions this strain synthesizes only benzylpenicillin. It is preserved as a freeze-dried preparation of spores.

Sporulation medium. Spores are produced on molasses agar medium of the following composition: glycerol, 0.75 %; molasses, 0.75 %; yeast extract, 0.5 %; NaCl, 4.0 %; MgSO₄·7H₂O, 0.005 %; KH₂PO₄, 0.006 %; ferric tartrate, 0.00016 %; CaSO₄, 0.025 %; CuSO₄ (1 % solution), 0.01 %; Bacto-agar, 3.0 %; pH value adjusted to 7.1. The medium is layered at least 0.5 cm. deep in plugged 20 oz. medical flats.

Method. Freeze-dried spores are taken up in a little Hartley beef broth and sown by spreading on the surface of the molasses agar. The bottles are incubated, medium uppermost, at 22° for 10 days. The cultures may then be stored in a cold-room (+2°) for at least 1 month.

Spores are separated from the mycelial mat by wetting the surface of the solid culture with 5 ml. 1 % aqueous 'Teepol' followed by washing with glass-distilled water and dilution to 250 ml. The resultant 'standard spore suspension' is used either as an inoculum for penicillin production (see below) or as a source of further freeze-dried preparations. For freeze-drying the spores are spun down, resuspended in about 10 ml. of a mixture made from equal volumes of inactivated horse serum and broth, and distributed in suitable ampoules.

Biosynthesis of penicillin

Medium. The organism is grown in a medium containing corn-steep solids, 2.0 %; lactose, 4.0 %; NaNO₃, 0.3 %; MgSO₄, 0.025 %; CaCO₃, 0.30 %; phenylacetic acid, 0.25 %; lard oil, 0.2 %; pH value adjusted to 5.6 before autoclaving; to this is added a 2 % (v/v) inoculum of 'standard spore suspension' and an aqueous solution of 'carrier-free' ³⁵S as ³⁵SO₄ in a volume not greater than 5 % of the total volume of medium.

Conditions of growth. A 250 ml. Erlenmeyer flask, capped with a 50 ml. beaker, is used as the growth vessel. To prevent rattling and permit free gas exchange the beaker is held clear of the neck of the flask by a collar made from three or four short pieces of pressure tubing threaded on wire. The flask, containing 40–50 ml. of medium, is swirled at 100 r.p.m. with a throw of 5 in. for 5–5½ days at 22° ($\pm 1^\circ$). No significant quantity of volatile radioactive compound is released during growth.

Yield. The concentration of penicillin in the harvested culture fluid is generally 750–950 units/ml. (by cup-plate assay). The proportion of added ^{35}S converted to labelled penicillin depends on the yield and the batch of corn-steep liquor used. At best the conversion rate is about 20 %.

Isolation of benzylpenicillin

The culture is brought to pH 7.0 with N-HCl , and filtered through a thin layer of 'Hyflo Supercel'. The penicillin is extracted from the filtrate by three successive liquid-liquid partition separations followed by chromatography on a column prepared and checked before the isolation is started.

Preliminary purification. (i) The filtrate is saturated with the minimum excess of 'Analar' $(\text{NH}_4)_2\text{SO}_4$ and the pH brought to 2–2.5 with 50 % phosphoric acid using thymol blue as external indicator. It is then extracted twice with one-fifth of its volume of 'Analar' amyl acetate and the aqueous phase rejected.

(ii) The combined amyl acetate phases are extracted twice with one-tenth of their volume of one-eighth saturated phosphate buffer, pH 6.5–7.0. The amyl acetate phase is rejected.

(iii) The combined aqueous phases are again acidified with 50 % phosphoric acid and extracted three times with one-half their volume of anaesthetic ether. The aqueous phase is rejected and the combined ether phases concentrated, if necessary, by evaporation with a stream of dry air. The final concentrate used for chromatography should contain about 5 mg. penicillin/ml. (assuming a 75 % yield so far).

Chromatography. The column described below operates satisfactorily with loads of up to 15 mg. of penicillin. Since optimal pH value and saturation of buffer increase with increasing column size, the dimensions given here are important.

7.5 g. of 'Hyflo Supercel' are thoroughly mixed with 3.75 ml. 25 %-saturated citrate buffer, pH 5.8. The material is packed 'dry', in about ten portions, into a tube of 1.1–1.2 cm. internal diameter, to give a column 18–20 cm. long.

The column is tested with a load of 15 mg. of pure benzylpenicillin in 3 ml. of ether, extracted from buffer by the process of (iii) above. The penicillin is washed through the column with anaesthetic ether saturated with the citrate buffer. Separation is unimpaired by operating at rates up to 3 ml./min. under a positive pressure of about 10 cm. Hg. Fractions of 3–4 ml. are collected in tubes containing about half the fraction volume of 0.0025 % bromthymol blue in neutral water. The fractions are titrated with vigorous agitation using 0.006 M-NaOH (1 ml. \equiv 2 mg. benzylpenicillin) from a CO_2 -free automatic

burette. The plotted titration results show a single large peak, spread over about 25 ml. between the 45th and 80th ml. of effluent, which corresponds to benzylpenicillin. The exact position of this peak is characteristic of the particular column in use.

The tested column is now used for the partially purified sample of ³⁵S-penicillin. Load and operating conditions are unchanged, but phenylacetic acid and other non-penicillin compounds give an unimportant preliminary peak in the 12–18 ml. range. The fractions known to contain benzylpenicillin are collected in a vessel cooled in ice + salt freezing mixture. About 10 ml. of glass-distilled water are added to the vessel and the penicillin titrated, with vigorous shaking, using 0.006 M-NaHCO₃, until the pH value remains unchanged at 7.5 (bromthymol blue as external indicator). The aqueous phase is then separated from the ether, distributed in lightly plugged ampoules and freeze-dried. The product is sodium benzylpenicillinate contaminated mainly with sodium citrate and appears quite stable when stored under dry N₂ at atmospheric pressure. The final yield is 60–70 % of the total penicillin in the original culture.

Assays and tests for purity. The antibiotic activity contained in each ampoule is determined, when it is opened for use, by a cup-plate assay. Freedom of the product from radioactive contaminants is checked by the paper chromatography method of Glister & Grainger (1950) combined with radioactivity measurements (Lester Smith & Allison, 1952). Duplicate strips run against mixed penicillin standards show no antibiotic activity other than that due to the benzylpenicillin spot, which also exhibits 85–90 % of the total radioactivity of the strip. Almost all the remaining radioactivity is found at the origin, and is probably due to penicilloic acid produced by the decomposition of penicillin on the strip itself (Lester Smith & Allison, 1952).

I am particularly indebted to Mr A. S. Stroud of Boots Pure Drug Co. Ltd., Nottingham, for providing most of the information on which the isolation procedure is based; and to Dr M. Lumb, also of Boots Pure Drug Co. Ltd., for supplying the organism and details of the culture media.

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(Received 8 August 1952)

Genetic Recombination without Sexual Reproduction in *Aspergillus niger*

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SUMMARY: Roper's technique for the production in filamentous fungi of strains with heterozygous diploid nuclei in their hyphae (Roper, 1952) has been applied successfully to *Aspergillus niger*, in which a sexual cycle does not occur. The diploids, heterozygous for known markers, give origin to new strains, most still diploid, homozygous for some or all of the markers and therefore associating or recombining in all possible ways the properties of the two strains from which the diploid was formed. Genetic recombination has thus been achieved in a filamentous fungus without a normal sexual cycle. Imperfect fungi are now open to genetic investigation. Deliberate 'breeding' of strains has become a practical proposition in industrial fermentations based on these fungi.

A technique developed by Roper (1952) made it possible to produce in the homothallic *Aspergillus nidulans* strains carrying in their hyphae and uninucleate conidia diploid nuclei heterozygous for known genetic markers. These diploid strains remained diploid through vegetative reproduction (hyphae and conidia) but yielded regularly, as a consequence of mitotic segregation and recombination, new strains homozygous for one or more of the markers (Pontecorvo & Roper, 1952*a, b*). The genetics of *A. nidulans*, which has a normal sexual stage in the life cycle, has been worked out in considerable detail (Pontecorvo, 1952*b*), and it was therefore possible in that species to study mitotic recombination in the light of this knowledge (Pontecorvo & Roper, 1952*b*; Pontecorvo, 1952*b*). However, Pontecorvo & Roper (1952*a*) suggested that the synthesis of diploid strains and the use of mitotic recombination would make it possible to carry out both genetic analysis, of a novel but perfectly valid type, and deliberate 'breeding' in species without a sexual stage. The first attempt in this direction, with *A. niger*, was successful (Pontecorvo, 1952*a*). The present paper reports details of the preliminary work done by Pontecorvo and additional results obtained since.

The production of heterozygous diploid strains

Unless explicitly stated, the techniques used in the present work were the same as those used as a routine in the extensive work with *A. nidulans* (Pontecorvo, 1952*b*).

Strains. Two strains, kindly supplied by Mr J. L. Yuill, were used throughout. They were both isolated by Mr Yuill after ultraviolet treatment of *A. niger* 680, a strain which he had isolated from Indian tea: 680A, mutant with split heads, as typical of the wild type, and fawn-coloured conidia; 680F, mutant with giobose heads and yellow coloured conidia, turning dark olive on ageing.

Both mutants were like *A. niger* 680 ('wild type') in nutritional requirements, i.e. they grew well on Czapek-Dox or similar media. Strains with these nutritional properties will be referred to as 'prototrophs'.

Media. Complete Medium (CM): a complex peptone, yeast extract, casein medium as used for *A. nidulans*. Minimal Medium (MM): a glucose, nitrate, inorganic salts medium as used for *A. nidulans*. Both strains grew well on these media. Incubation was at 28°; 48 hr. after plating the colonies are classifiable as to colours of conidia.

Isolation of nutritional mutants. Conidial suspensions in saline-Calzolene were well broken up and adjusted by dilution to a density of 10,000 or 5000/ml.; 0.1 ml. of suspension was spread on the surface of each of a number of CM dishes and exposed for 6 min. with the lids removed to ultraviolet from Hanovia XI low-pressure mercury lamp at 30 cm. distance. After incubation a random sample of about 200 well-separated colonies was isolated and the 200 strains were passed through the routine tests for identifying and characterizing nutritional mutants (Pontecorvo, 1952*b*). The results of one series from each of the two strains are shown on Table 1.

Table 1. *Ultraviolet irradiation of strains 680 A (fawn) and 680 F (olive) to obtain nutritional mutants*

Strain	Conidia plated		Colonies developed		No. of colonies isolated	No. of nutritional mutants from isolates
	Total no.	No./dish	No.	% plated conidia		
680 A (fawn)	8000	1000	587	7.3	197	3
680 F (olive)	4500	500	668	14.8	200	2

The lower survival, after irradiation, of conidia of 680 A is probably due to their visibly thinner cuticle.

The five nutritional mutants were given the symbol A or F, according to the parent strain from which they derived, followed by a code number. Besides having the morphological characters of the parents, i.e. fawn conidia—split heads, or olive conidia—globose heads, respectively, the new mutants had the following nutritional properties:

A1: requiring thiamine or its 'thiazole'; forming compact colonies with somewhat reduced growth rate.

A33: requiring arginine. Citrulline, ornithine and proline ineffective. Competitively inhibited by lysine.

A35: requiring guanylic acid, guanosine or guanine. Adenosine and adenine very slightly effective.

F92: requiring histidine.

F104: requiring casein digest. Casein hydrolysate ineffective.

These five strains were purified by single colony isolation.

Formation of balanced heterokaryons. Heterokaryotic heads, of colours differing from those of the strains used and approaching that of the wild type, are known to arise in *A. niger* when pairs of mutant strains, differing from one another and from the wild type in the colour of the conidia, are grown in

mixed culture (Gossop, Yuill & Yuill, 1940). Mixed cultures of the prototrophs 680A (fawn) and 680F (olive) confirmed this finding. A few heterokaryotic heads ranging in colour from slightly darker than those of either parent strain to as black as those of the wild type were regularly found. The chains of conidia in individual heterokaryotic heads were uniform in colour. From the fact that the chains of conidia in heterokaryotic heads were not of either parental colour, and that those in one head tended to be uniformly different from both parental types, we deduced (Pontecorvo, 1947) that the reactions blocked in the mutants involved diffusible substances. This is quite different from the situation in the white and yellow mutants of *A. nidulans*, where the reactions are cell localized (Pontecorvo, 1952*b*).

The production of further mutational differences mentioned in the preceding section made it possible to synthesize 'balanced' heterokaryons (Pontecorvo, 1947) differing from the component strains not only in colour and morphology of the conidial heads but also in nutritional requirements: i.e. able to grow on MM on which the component strains cannot grow singly.

The following balanced heterokaryons were synthesized in the usual way (Pontecorvo, 1952*b*) and kept by hyphal tip transfers on MM: A1 (fawn, thiamineless)+F92 (olive, histidineless); A35 (fawn, guanosineless)+F92 (olive, histidineless); A33 (fawn, arginineless)+F104 (olive, requiring casein digest).

A majority of the heads of these heterokaryons grown balanced on MM were heterokaryotic and ranged in colour and morphology from wild type (black, split) to the types of the two component strains (Pl. 1, fig. 1).

Macroscopically, a balanced heterokaryotic colony shows a uniform growth on MM with peppery colour due to the thorough mixture of black, intermediate, fawn and olive heads. That the heads differing in colour and morphology from those of either component strain were actually heterokaryotic, was shown by the fact that individual conidia from them would give rise only to colonies of either component type, although conidia of both kinds were produced by one and the same head. It must be remembered that the conidia of *A. niger* are uninucleate (Yuill, 1950).

Isolation of diploids. Both techniques described by Roper (1952) were used, i.e.:

(1) Plating of conidia of balanced heterokaryons on to MM. On MM the conidia of each parental type cannot give origin to colonies because they require either of two growth factors. Diploid conidia originated from fusion of two unlike nuclei, on the other hand, being heterozygous and therefore presumably non-requirer, should grow and presumably give origin to dark-spored colonies.

(2) Allowing the heterokaryon to grow on MM and searching for sectors or spots showing exclusively black growth instead of the mixture of olive, fawn, black and intermediate. Camphor treatment of the heterokaryon was carried out as described by Roper.

Diploids were obtained from all three mentioned balanced heterokaryons: A1 + F92, diploids obtained from sectors, both in camphor-treated and control

heterokaryons; A35 + F92, diploids obtained from plating, both from camphor treated and controls; A33 + F104, diploids obtained from sectors, without treatment.

To distinguish them from heterokaryons these diploids will be indicated by using, above and below the fraction sign, the symbols of the two strains from which they originated.

Diploids seem to arise more easily, even without camphor treatment, in *A. niger* than in *A. nidulans*. In the case of A35 + F92 a comparison of the frequency of heterozygous diploid conidia arising in the balanced heterokaryon with and without camphor treatment was carried out (Table 2).

Table 2. Comparison of the proportions of heterozygous diploid conidia present in heterokaryons A35 + F92 with and without camphor treatment

	No. of plated conidia	Diploid colonies	
		No.	Per 10 ⁷ conidia
From a treated heterokaryon	1,800,000*	8	46
From a control heterokaryon	8,700,000	3	3.5

* Corrected for viability, which was 40 %. Plating at two densities: 10⁶ and 10⁸, per dish.

Taken at their face value the results of Table 2 suggest that camphor is effective. Fluctuations between the control cultures due to the clonal distribution of diploid nuclei, however, must be enormous. For this reason, and others discussed by Pontecorvo & Roper (1952*b*), it would be premature to draw conclusions. From the practical point, camphor treatment was not essential with the strains of *A. niger* used for obtaining the diploids.

All three types of diploid kept for further work were derived from non-treated heterokaryons and purified by isolation of a single conidium by means of a micromanipulator.

Properties of diploids. The diploids differed from the heterokaryons and from the haploid parent strains in a number of ways. First, they had heads almost as black as those of the original *A. niger* 680, instead of heads of a whole range of colours like the balanced heterokaryons (Pl. 1, fig. 1), or heads uniformly fawn or uniformly olive like either parent strains. Secondly, they could grow on minimal medium like the heterokaryon but unlike either parent strain. Thirdly, on medium supplemented with either or both of the growth factors required by the parent strains, they would continue to produce uniformly dark heads as they grew, instead of segregating out into fawn and/or olive sectors like the heterokaryon. Fourthly, they had conidia about 1.3 times the diameter of *A. niger* 680 (Table 5; Pl. 1, figs. 3 & 4), i.e. about double the volume. Fifthly, they would produce rare fawn or olive heads (Pl. 1, fig. 2), or small spots of heads, or sectors of these two parental colours. Similarly to the results with *A. nidulans* (Pontecorvo & Roper, 1952*b*; Pontecorvo, 1952*a*), most of the strains ('segregants') established by isolating from these fawn and olive spots were still demonstrably diploid. The properties

of these segregants, and of those showing either or both thiamine and histidine requirement (see further), support the inference, inescapable in the case of *A. nidulans*, that they originated as a consequence of mitotic segregation and recombination, probably by a mechanism of mitotic crossing-over as demonstrated by Stern (1936) in *Drosophila*.

Segregation and recombination

Colour segregants. All the work on mitotic segregation was carried out on diploid A1/F92. With the diploids A35/F92 and A33/F104 we limited ourselves to verifying that they too produced occasional fawn or olive spots.

Isolation, from colonies of A1/F92 growing on CM, of single fawn or olive heads from each spot was carried out by hand under the dissecting microscope. Each isolate was purified by streaking and, when necessary, by further single conidium micromanipulation. At the beginning of this work cultures of A1/F92 used for this isolation were from stab-inocula of masses of conidia at two points in each Petri dish. Later it was found preferable to plate about 100 conidia per dish. In this case not more than one fawn, and/or one olive spot per colony was isolated; the danger of isolating more than once from one and the same 'segregant' clone was thus avoided. One or two fawn spots are usually found on the average in each colony of 5 mm. in diameter; the olive spots are much rarer.

The purified segregants were plate-tested for nutritional requirements in the routine way (Pontecorvo, 1952*b*). Every one of the 115 fawn or olive segregants reported in this paper and isolated as mentioned, or isolated by means of the SO₂ technique (see p. 203), required one, the other, both or neither of the two growth factors required by A1 (thiamine) and F92 (histidine), respectively. No requirement not present in either parent strain arose in any one of the fully tested 1727 isolates (including the above 115) mentioned in the present paper.

Table 3 shows the classification of sixty-one fawn or olive segregants isolated by hand from colonies of A1/F92. The majority were prototrophs, differing from A1/F92 in colour but not in requirements and from the original strains (A1 and F92) in requirements but not in colours. Two segregants were like A1 and F92, respectively, both in colour and requirements; and one was a 'recombinant' because it had the fawn colour of A1 and the histidine requirement of F92.

Far more fawn than olive segregants were isolated. This reflects both the greater ease with which fawn heads are noticed and their greater frequency in colonies of A1/F92. The incidence of segregants was estimated by plating conidia of A1/F92 and classifying the resulting colonies for colours and requirements (Table 3A). Out of 651 colonies, three were fawn prototrophs and two dark thiamineless. Plating of conidia of two dark segregants—26 (7) histidineless, and 12 (16) aneurineless (Tables 4 and 5), both known to give colour segregants—gave no fawn or olive out of 656 colonies. In all, therefore, segregants for fawn constitute about one in a few hundred conidia and segregants for olive are less frequent than this.

An accurate estimate of the frequency of segregation (as distinct from the proportion of segregant conidia) would require, of course, very careful work, including serial isolation of conidia in individual chains.

The classification of the sixty-one colour segregants selected visually (Table 3) and of the 651 colonies from plated conidia of A1/F92 showed, as

Table 3. 'First-order' segregants from diploid *Aspergillus niger*:

		A1 (<i>fawn, thiamineless</i>)		F92 (<i>olive, histidineless</i>)	
	Prototrophs	Requiring thiamine	Requiring histidine	Requiring both	Total
A. Unselected*					
Dark	646	2	0	0	648
Fawn	3	0	0	0	3
Olive	0	0	0	0	0
	649	2	0	0	651
B. Selected					
(1) For colour†					
Fawn	54	1	1	0	56
Olive	4	0	1	0	5
	58	1	2	0	61
(2) For requirements‡					
Dark	591	13	9	0	613
Fawn	4	9	0	0	13
Olive	7	0	4	0	11
	602	22	13	0	637

* Conidia of A1/F92 were plated on CM, and 651 colonies were classified as to colours and requirements.

† Fawn and olive heads, isolated from spots in A1/F92 colonies, were purified and classified as to requirements.

‡ 'Auxotroph enrichment' by SO₂ treatment. Conidia of A1/F92 pre-germinated in MM, were treated with SO₂ and plated on CM. 637 colonies were classified as to colours and requirements.

expected from the work with *A. nidulans*, that in heterozygous diploids of *A. niger* segregation occurs both for colour and nutritional markers, and the various markers also recombine. It was then necessary to investigate more extensively which types of segregants and recombinants could be obtained from A1/F92.

Nutritional segregants. The identification of colour segregants is carried out by inspection. Even though fawn or olive heads constitute perhaps only 1/1000 of the total, they stand out clearly among the mass of parental (dark) heads and they can be isolated easily by hand (Pl. 1, fig. 2). Segregants for nutritional requirements, on the other hand, cannot be identified by inspection and the problem is therefore that of selecting otherwise than visually.

A technique, in the process of being developed by Forbes (1952), has been used to this purpose. It is based on the fact that SO₂ is more toxic to germinating conidia than to dormant ones. If a mixture of conidia, some prototrophs and some requiring a growth factor, is incubated in MM, almost 100% of

the former and none of the latter germinate within 6 hr. at 37°. Treatment with SO₂ of this pre-germinated suspension kills off preferentially the prototrophs, and therefore increases the proportion of requirers ('auxotrophs') among survivors ('auxotroph enrichment'). This technique is still far from standardized, and therefore no quantitative meaning can be attached to its

Table 4. 'Second-order' segregants from first-order segregant 26 (7), dark histidine-requiring

	Requiring histidine	Requiring histidine and aneurine	Total
A. Unselected*			
Dark	211	0	211
Fawn	0	0	0
Olive	0	0	0
	211	0	211
B. Selected			
(1) For colour†			
Fawn	26	0	26
Olive	1	0	1
	27	0	27
(2) For requirements‡			
Dark	139	1	140
Fawn	0	0	0
Olive	0	0	0
	139	1	140

* Conidia were plated on CM and the derived colonies classified as to colours and requirements.

† Single fawn or olive segregant heads were isolated from colonies obtained as in footnote above, not more than one head from each colony.

‡ 'Auxotroph enrichment' by differential centrifugation. Conidia were pre-germinated for 8 hr. in liquid MM supplemented with histidine. The suspension was then centrifuged at low speed, and the supernatant plated on CM. 139 colonies were classified as to colours and requirements and one (heavy type) was of the wanted type.

results (Table 3B (2)): it has led, however, to the isolation of types of segregants and recombinants which it would have been very laborious to isolate without enrichment.

The technique was applied to A1/F92 conidia. A suspension (from a 4-weeks-old stab-inoculum culture on CM) of 6.6×10^5 conidia/ml. was incubated in liquid MM in a water bath at 38°. After 7 hr. about 90 % of the conidia showed germination tubes; 8 ml. of water were added to 1 ml. of pre-germinated suspension, and to this 1 ml. of SO₂ solution was added. After 8 min. at room temperature the SO₂ was oxidized with KMnO₄ (Forbes, 1952), and plating was carried out with 0.2 ml. 13,200 conidia/Petri dish of CM. In twenty-three Petri dishes, 802 colonies came up, i.e. about 8/1000 plated conidia. 687 colonies were isolated and tested with the results shown in Table 3B (2).

As control a sample of the original suspension was plated on fifteen

dishes of CM, at the density of sixty-six conidia per dish. A total of 666 colonies came up (about 70 % of the plated conidia) and of these 651 were isolated and tested with the results shown in Table 3A. Clearly the SO_2 technique enriched at least tenfold the proportion of requirers.

Among the recombinants for two or more of the properties in which A1 and F92 differed certain classes were not recovered from this experiment. There were neither double-requirers (thiamine and histidine) in any colour combination nor any new colour type which could be the fawn-olive double mutant, nor olive thiamine-requiring, nor fawn histidine-requiring. The last type, however, had been already obtained among the segregants selected for colour.

Seven of the dark segregants—five requiring thiamine and two histidine—obtained by auxotroph enrichment were purified by single conidium micro-manipulation and plated to see whether the resulting colonies would still give origin to colour segregants. All but one [15 (16), Table 5] segregated for either or both of the colours. We shall call 'first-order' segregants those originating directly from A1/F92, 'second-order' and 'third-order' segregants those originating from 'first' or 'second-order' segregants respectively.

By isolation of a single olive head from a dark thiamineless first-order segregant [12 (16), Table 5], an olive thiamineless second-order segregant (OV, Table 5) was secured, a type not previously obtained.

To obtain other missing types of segregants a further auxotroph enrichment treatment was carried out on strain 26 (7) (Table 5), a first-order dark histidineless which segregated for fawn and olive. This time the enrichment was based on differential centrifugation. Conidia of 26 (7) were incubated as for the SO_2 treatment, but in MM supplemented with histidine. After 8 hr. the suspension was centrifuged at 1000 r.p.m. for 2 min. Most of the germinated conidia were in the centrifugate, while a high proportion of non-germinated ones were in the supernatant. The supernatant was plated on five dishes of CM at a density of 150 conidia per dish. 149 colonies, all dark, developed out of the estimated 750 conidia plated; of those 140 were isolated and tested (Table 4B (2)): 139 required histidine like the parent strain, but one [3 (1), Table 5] was of the wanted type, i.e. thiamine- and histidine-requiring. Control platings of conidia of 26 (7) gave only dark histidineless colonies like 26 (7) [Table 4A]. Visual isolation of twenty-six fawn and one olive segregant from 26 (7) again gave only histidineless strains (Table 4B (1)).

The second-order dark, thiamine- and histidine-requiring strain [3 (1)], obtained as just mentioned by auxotroph enrichment segregated for fawn and olive: isolation of one segregant of each colour led to the establishment of third-order segregants: a fawn, thiamine- and histidine-requiring (FHV, Table 5), and a similar olive strain (OHV, Table 5).

With these isolations the set of strains associating the three known colours and the four nutritional types in all possible ways was completed. The twelve different combinations obtained were those of either dark, or fawn, or olive with thiamine requirement, histidine requirement, both requirements and neither, respectively.

Clearly, one group of recombinants was still missing: i.e. the one with both fawn and olive mutant markers. However, five and perhaps all, of the six olive segregants of Table 5, produce light olive spots with split heads instead

Table 5. *Mean diameter of conidia from different haploid, diploid, and segregant strains of Aspergillus niger*

Strain	No. of conidia measured	Mean diameter (μ .)	Strain	No. of conidia measured	Mean diameter (μ .)
STARTING STRAINS			SEGREGANTS FROM A1/F92		
Dark			Dark		
680, prototroph	52	4.27	26 (17)a, prototroph*	100	5.62
	100	4.06	32 (8), thiamineless*	100	4.20
	100	4.12	29 (15a), thiamineless*	100	4.16
	100	4.14	9 (17)b, thiamineless*	100	4.54
Fawn			15 (16), thiamineless	100	3.84
680 A, prototroph	60	3.70	12 (16), thiamineless	100	4.76
	100	3.58	23 (7), histidineless	100	5.38
A1, thiamineless	100	3.58	26 (7), histidineless	100	5.46
A33, arginineless	100	3.76	3 (1), thiamine- and	100	5.10
A35, guanosineless	100	4.04	histidineless		
Olive			Fawn		
680 F, prototroph	64	4.69	29 (15)b, prototroph	79	3.65
	100	4.40		100	3.60
F92, histidineless	100	4.32	II/33f, prototroph	100	4.46
F104, casein digestless	100	4.22	I (1), thiamineless	100	3.32
SYNTHESIZED DIPLOIDS			4 (2)b, thiamineless	100	3.62
Dark			6, histidineless	86	4.00
A1/F92, prototroph	53	5.39		100	3.62
	100	5.26	FHV, thiamine- and	100	4.80
	100	5.24	histidineless		
	100	5.28	Olive		
A35/F92, prototroph	58	5.31	II/33, prototroph	62	5.48
	100	5.46	29 (15)c, prototroph	100	4.62
A33/F104, prototroph	100	5.36	OV, thiamineless	100	5.16
			I/11, histidineless	100	4.42
			15 (5)c, histidineless	100	4.70
			OHV, thiamine- and	100	5.14
			histidineless		

The means based on 100 conidia are for CM cultures, mainly 7-15 days old, measuring to the nearest division of the eyepiece micrometer five conidia in a chain. With the magnification used, 1 div. = 2 μ .; the maximum error of any one measurement is thus about 10 %. The means based on less than 100 conidia are from earlier measurements with non-standardized age and media, and taking chains of from three to ten conidia. The greatest discrepancy between two means for the same strain is, nevertheless, only 0.38 μ . (strain 6).

* Four strains, one prototroph and three thiamineless, with heads considerably darker than those of A1/F92.

of globose. One of these six olive segregants (II/33) produces in addition spots indistinguishable both in morphology (split) and colour from fawn (isolated as strain II/33f).

Are either of these light olive spots or the fawn spots, produced by olive strains, the missing type of colour recombinant? If the former were the missing type it would mean that the interaction of fawn and olive gives an intermediate colour, i.e., light olive. If the latter were the missing type, we

would have an example of 'epistasis' similar to that found in *A. nidulans* between white, yellow and green (Pontecorvo, 1952*b*).

A number of genetic tests which could decide this question have already been devised, they include a thorough search for light olive second-order segregants from first-order fawn segregants; and an investigation of the colours of heterokaryotic heads produced by various combinations of light olive, fawn and olive strains.

Table 3 reveals a fact already pointed out by Pontecorvo (1952*a*); i.e. that the distribution of simultaneous segregations for more than one marker is not at random among segregants. In summary:

Colours	Among nutritional segregants		Nutritional properties	Among colour segregants	
	Thiamineless	Histidineless		Fawn	Olive
Fawn	10	1	Thiamineless	10	0
Olive	0	5	Histidineless	1	5
All colours	25	15	All types	72	16

In the present case, how much of this great excess of simultaneous segregation for more than one marker is due to linkage and how much to segregation occurring wholesale in certain nuclei will have to be investigated.

Properties of segregants. Table 5 gives the diameter of the conidia of the original strain, the mutants derived from it, the three synthesized diploids, and a sample of segregants of first, second and third order from A1/F92. There is no overlap between the diameters of the conidia of the synthesized diploids and any of the haploid strains, though there is considerable variation in the diameters of the latter, the olive strains having larger conidia than the fawn. As shown by replicates of measurements of the same strain under different conditions, the diameter of the conidia seems to be a rather constant strain character.

The synthesized diploids have diameters of conidia almost exactly 1.3 times that of *A. niger* 680, i.e. a volume almost exactly double. On the other hand, the diameters of the segregants cover a continuous range, from that of the smallest haploid to that of the largest diploid. Clearly in *A. niger* the diameter of the conidia is not very significant for identifying diploid strains whenever a number of known and unknown genetic differences are segregating and recombining. By conidial size alone, we cannot tell which segregants are still diploid, like A1/F92 from which they derived by one or more steps, and which are haploid. Tests of further segregation, however, can give a one-way answer in some cases.

If a segregant for one of the markers, say a requirement, still segregates for one or more of the remaining markers for which A1/F92 was heterozygous, we can conclude that it is a diploid (or perhaps a higher polyploid). However, if a segregant does not segregate further, we cannot conclude that it is haploid, because it could be a diploid homozygous at all relevant loci. As segregation for colour markers is detected visually, and for requirements by laborious tests, we have limited ourselves to the former for most of the segregants of Table 5. In the case of fawn strains, no further colour segregation has been

detected so far. In the case of olive strains, as already stated, five and perhaps all six investigated segregate for light olive: the precise nature of these light olive putative segregants will have to be established. In the case of the nine dark segregants of Table 5, eight give further colour segregation; three for fawn and olive, four for fawn only, and one for olive only. The one which does not segregate further—15(16)—incidentally, is the one with the smallest conidia.

It is certain, thus, that a high proportion of dark segregants are still diploid and the same is probably true of the olive segregants. As to the fawn, a way to detect diploidy would be a search for nutritional second-order segregants.

Genetic analysis. Mitotic segregation and recombination makes it possible to analyse genetically our strains even though the precise rules of the game are as yet unknown. Our original strains A1 and F92 differed in the following properties from one another and from the wild type:

Morphology: split heads versus globose heads, the wild type having split heads.

Colour: fawn versus olive, the wild type having dark heads.

Requirements: thiamine requirement versus histidine requirement, the wild type having neither.

We have therefore a minimum of five pairs of alternative properties: split/globose; fawn/dark; olive/dark; thiamine requirement/thiamine independence; histidine requirement/histidine independence.

All of these differences are under genetical control because the heterokaryons between A1 and F92 show properties different from those of either strain and approaching those of the wild type, but single conidia from heterokaryotic heads give origin again to unchanged A1 or F92. Furthermore, every one of these differences segregates vegetatively in the heterozygous diploids, and recombination between all but two of them has been obtained. The two which so far have not been unquestionably recombined are split/globose and olive/dark: we have not obtained globose fawn or globose dark types, globose recombinants being all olive, though light olive strains with split heads arise from most globose olive strains.

Leaving for the moment the question of whether globose and olive are recombinable differences, i.e. due to mutation at different loci, we have thus identified at least four different loci, recombination between which occurs readily. These loci may be symbolized by pairs of alleles as follows: *A/a*, normal versus fawn colour; *O/o*, normal versus olive colour; *THI/thi*, independence of, versus requirement for thiamine or 'thiazole'; *HIST/hist*, independence of, versus requirement for histidine.

Using the capital symbols for the alleles of the wild type (clearly all dominant both in the heterokaryon and the diploid) we may indicate the tentative genotypes of our starting strains and of diploid A1/F92 as follows:

<i>A. niger</i> 680	<i>A</i>	<i>O</i>	<i>THI</i>	<i>HIST</i>
680A	<i>a</i>	<i>O</i>	<i>THI</i>	<i>HIST</i>
680F	<i>A</i>	<i>o</i>	<i>THI</i>	<i>HIST</i>
A1	<i>a</i>	<i>O</i>	<i>thi</i>	<i>HIST</i>
F92	<i>A</i>	<i>o</i>	<i>THI</i>	<i>hist</i>
A1/F92	<i>a/A</i>	<i>O/o</i>	<i>thi/THI</i>	<i>HIST/hist</i>

When it comes to those segregants and recombinants which are certainly still diploid (because they segregate further) we have several possible genotypes for most of the phenotypes. For instance, as to colours:

	Fawn		Olive		Dark	
Genotypes:	a/a	O/O	A/A	o/o	A/A	O/O
	a/a	O/o	A/a	o/o	A/A	o/O
	a/a	$o/o?$	a/a	$o/o?$	A/a	O/O
					A/a	o/O

As to requirements, diploid segregants *not* requiring a growth factor may be of two genotypes: homozygous for the dominant allele or heterozygous. In all, therefore, there are at least $3^4 = 81$ different genotypes, but probably only 12, or possibly 16, distinguishable phenotypes: 12 have already been obtained.

All of the genotypes could, in theory, be distinguished by appropriate genetic tests; one kind of test is that of further segregation, which is easy enough in the case of colour markers and which we have in fact carried out on some segregants. Another test is that of finding out the kinds of phenotype produced in heterokaryons. This we have carried out in three cases, between fawn and olive segregants, obtaining dark heterokaryotic heads in two cases but not in a third.

Other important problems are, of course, those of linkage. The data of Table 3 are already suggestive of something in this direction. A serious study of linkage, however, must wait until the process of mitotic recombination is better understood. For this purpose *A. niger* is unsuitable because of the impossibility of cross-checking the results of mitotic recombination with those obtained via sexual reproduction. The work in progress with *A. nidulans* is more promising (Pontecorvo & Roper, 1952*b*; Pontecorvo, 1952*a*). We shall be able to come back to *A. niger* as soon as the latter work has yielded some preliminary answer.

Conclusions

The present work shows that by synthesizing heterozygous diploid strains and investigating the recombinants produced mitotically by them, genetic analysis is possible in a species of filamentous fungus in which the standard sexual cycle does not occur. In the present preliminary work at least four loci have been identified on the basis of an equal number of recombinable differences. A search for linkage between any two or more of these, or of other, loci and the construction of chromosome maps is only a matter of extending the present work.

The approach described here, in addition, has very practical implications for those industrial fermentations which use filamentous fungi. By producing heterozygous diploids between two strains differing in properties, new *stable* strains can be obtained associating and recombining these properties or showing new ones. In the 'breeding' of improved strains of industrial moulds our methods have potentialities and limitations of the same order as those of hybridization in horticulture.

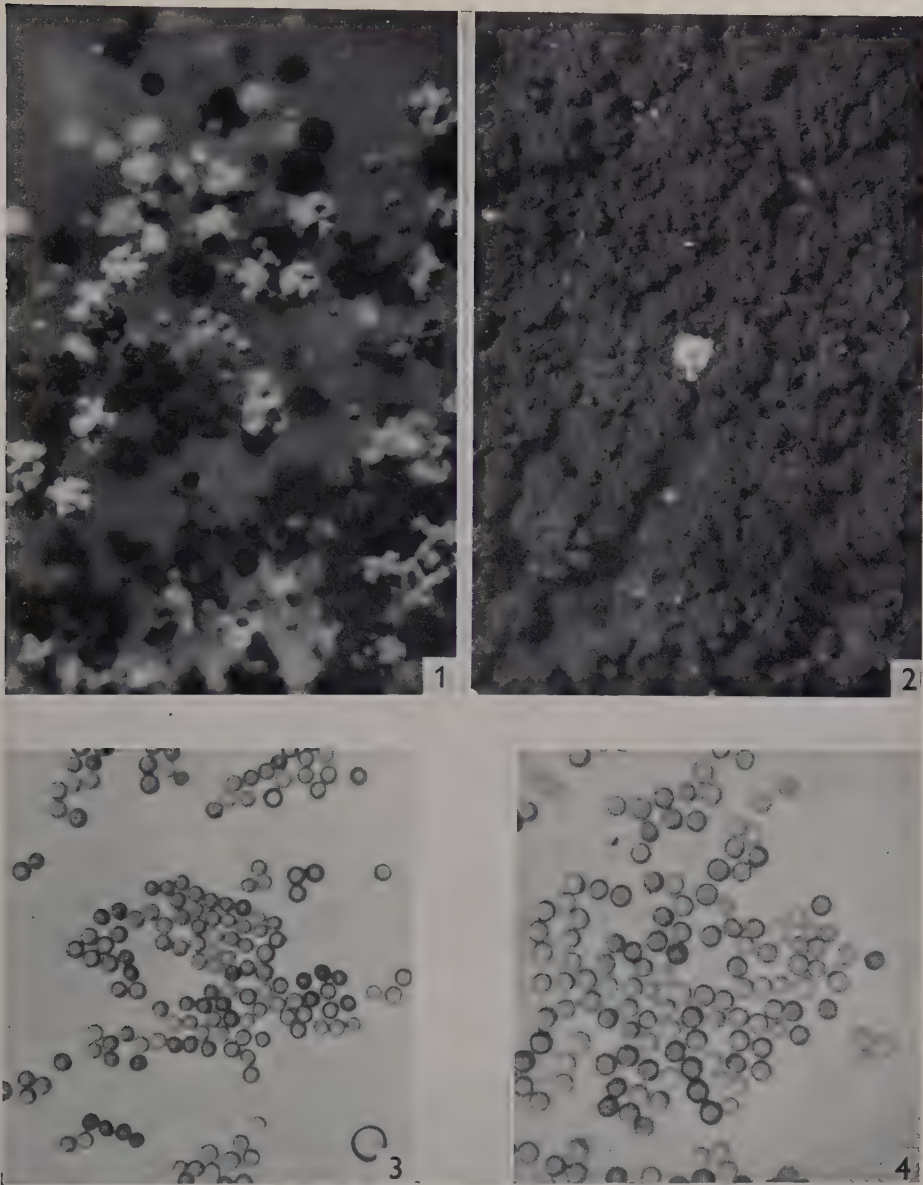
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EXPLANATION OF PLATE

- Fig. 1. Balanced heterokaryon between strains A1 (fawn, split heads, thiamine-requiring) and F92 (olive, globose heads, histidine-requiring). Note fawn split, olive small globose, black split heads and heads of intermediate shades and shapes.
- Fig. 2. Diploid A1/F92 formed from the same two strains as the heterokaryon in fig. 1. Almost all heads are black; the single fawn split head is a segregant.
- Figs. 3 & 4. Conidia of 11-day-old cultures on complete medium, $\times 500$. Fig. 3. Haploid *A. niger* 680. Fig. 4. Diploid A1/F92.

(Received 5 August 1952)



G. PONTECORVO, J. A. ROPER & E. FORBES—GENETIC RECOMBINATION. PLATE 1

SUSSMAN, A. S. (1953). *J. gen. Microbiol.* 8, 211-216.

The Effect of Heterocyclic and other Compounds upon the Germination of Ascospores of *Neurospora tetrasperma*

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SUMMARY: Eleven 5-membered heterocyclic compounds, in addition to furfural and furfuryl alcohol, were effective in overcoming the dormancy of ascospores of *Neurospora tetrasperma*. The substitution of a nitro group in the 5-position imparted fungistatic properties to otherwise innocuous furans and pyrroles; carboxylic acid derivatives of these heterocyclic compounds likewise were toxic. From data on the time-course of furfural activation, it is shown that the Q_{10} for this process is 4.1 over the temperature range from 20 to 30°.

The work of Shear & Dodge (1927) and of Goddard (1935, 1939) and Goddard & Smith (1938) established that ascospores of *Neurospora* spp. can be activated by heat treatment. No chemical means of inducing germination was known until Emerson (1948) showed that furfural and furfuryl alcohol are effective activators of such ascospores at concentrations as low as 1 part per million. That this effect is probably not an isolated one is suggested by the recent demonstration that furfural will also activate the spores of certain thermophilic bacteria (Mefferd & Campbell, 1951). In order to extend the spectrum of chemical activators of ascospores of *Neurospora* spp., and to explore the mechanism of their action, analogues of furfural, other heterocyclic compounds, and some miscellaneous compounds having physiological activity were tested for their effect on germination.

MATERIALS AND METHODS

Ascospores were obtained by crossing strains 374 and 377 of *N. tetrasperma* which were grown as outlined by Goddard (1935). Spores were used immediately after harvesting, since Emerson (personal communication) has shown that they gradually lose their sensitivity to furfural until, after 3 months, they become almost unresponsive to such treatment; this has since been confirmed by the present author.

Tests for activators were carried out by a modification of the method described by Davies, Duckworth & Harris (1948). After suspending the spores in 0.05 % (w/v) Nacconal (Allied Chemical and Dye Corp., New York, N.Y.) for a few minutes, they were rinsed 4 times by centrifugation in distilled water. Then the spores were collected on a sintered glass filter or in the bottom of centrifuge tubes and were placed in 25 ml. Erlenmeyer flasks to which had been added 5 ml. of the solutions to be tested. The flasks were shaken for 6-7 hr. at a rate of 80 oscillations/min. with a throw of 10 cm. at a temperature of 25°. At the end of this period of time it was usually found that germination had progressed to a point where the germ tubes were about 80 μ . long, whereupon formaldehyde was added to the flasks in order to kill

the spores. Two counts of at least 500 spores each were made and the average percentage germination determined; by this means an error below 5% was consistently obtained. A spore concentration of about 100,000 cells/ml. was used throughout.

Where the inhibitory effect of chemicals upon germination was observed, spores were washed as described previously but were then activated by heating for 30 min. at 60° in a constant-temperature oil-bath. Inoculations, incubation and counts were performed as before.

Testing of compounds was carried out using at least three dilutions, ranging from 1×10^{-2} M to 1×10^{-5} M. Solvents and some other substances were used in concentrations as high as 1×10^{-1} M. All compounds which were found to be active were tested for the presence of contaminant furfural by the aniline acetate test which is sensitive enough to detect up to 1 part per million of this compound. Easily polymerizable compounds like furfural, furfuryl alcohol and pyrrole were vacuum-distilled before use.

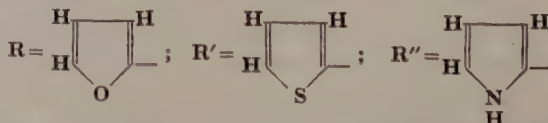
RESULTS

Chemical activators

As Emerson (1948) showed, furfural and furfuryl alcohol were active in breaking the dormancy of ascospores. In addition, the 11 other compounds listed in Table 1 were found to be active. The activation process shows a rather striking specific requirement for a 5-membered heterocyclic ring as repre-

Table 1. *Compounds which are active in breaking the dormancy of ascospores of Neurospora tetrasperma*

	Molar activity*	% of furfural activity	Formula	Concentration at which molar activity measured (M)
Furfural	248	100	RCHO	1×10^{-4}
2-Furfuryl alcohol	198	77	RCH ₂ OH	1×10^{-3}
2-Methyl furan	189	76	RCH ₃	1×10^{-3}
2-Furfurylmethylether	22	9	RCH ₂ OCH ₃	1×10^{-2}
2-Furfural acrolein	126	51	RCH-CHCHO	1×10^{-3}
2-Furfural diacetate	280	113	RCH ^{COOCH₃} _{COOCH₃}	1×10^{-4}
Thiophene	18	4	R'H	5×10^{-4}
2-Thiophene carboxaldehyde	198	80	R'CHO	1×10^{-3}
2-Thiophene chloride	214	86	R'Cl	1×10^{-3}
2-Thiophene bromide	94	38	R'Br	1×10^{-3}
Pyrrole	132	53	R''H	1×10^{-3}
2-Acetyl-pyrrole	18	8	R''COCH ₃	1×10^{-3}
Diethyl ether	41	16	C ₂ H ₅ OC ₂ H ₅	1×10^{-1}



* The 'molar activity' was computed by multiplying the percentage germination by the negative logarithm of the molar concentration of the compound used. The concentration giving the highest 'molar activity' was used in making the computations.

sented by the furans, thiophenes and pyrroles, with diethyl ether as an exception to this rule.

On the other hand, many related and other heterocyclic compounds which were tested showed no activity. These included: furan, 2-furoic acid, 2-furoamide, 2-furoyl chloride, *n*-propyl furoate, *iso*-amyl furoate, allyl furoate, 5-methyl-2-furoic acid, 5-bromo-2-furoic acid, 2-furfurilidene acetone, 2-furylacrylic acid, 2-furfuraldoxime, 2-furylmethylketone, 2-furfurylmercaptan, 2-nitrofuran, 5-nitro-2-furfural, 5-nitro-2-furfurylmethyl ether, furil, furoin, 3-furyl acrolein, tetrahydrofuran, 2-tetrahydrofurfuryl alcohol, 2-methyltetrahydrofuran, pyrrolidine, 2:5-dihydropyrrole, and pyridine. Moreover, the following compounds which have various known physiological activities were also ineffective in activating the ascospores: adenine, adenosine, adenylic-5-acid, adenosine triphosphate (ATP), thiamine, coumarin, nicotinic acid, riboflavin, imidazole, folic acid, glutathione, cysteine, cystine, indole-3-acetic acid, indole-3-propionic acid, nicotine, yeast extract, nucleic acid (yeast), casein hydrolysate (enzymatic), haemin, haemoglobin, ribose, glucose, L-proline, L-hydroxyproline, and thiazole. In all, over 120 substances were tested and, of these, only 13 (Table 1) proved to be active.

The activity of diethyl ether, although low, suggested that other organic solvents might be active. With this in mind, chloroform, acetone, light petroleum, various ethers (*n*-propyl to *n*-amyl) and alcohols (methyl to *n*-capryl) were tested but were all inactive. It was also determined that 7 parts/1000 (approx. 1×10^{-1} M) of diethyl ether was optimal for activation, a concentration which is far higher than that required for activation by the effective heterocyclic compounds.

Table 2. *Length of time required to immerse ascospores of Neurospora tetrasperma in furfural in order to effect 50 % activation*

Temperature	Time necessary to induce 50 % germination (min.)	Q_{10}
20°	34	4.1
30°	8	2.0
35°	5.5	

In order to determine the speed with which chemical activation is effected, 40 mg. of spores were suspended in 4 ml. of 5×10^{-4} M-furfural and were incubated at various temperatures. At intervals thereafter, 0.1 ml. samples were withdrawn, washed in 0.005 % (w/v) Nacconal, rinsed three times in distilled water (found to be sufficient to remove all measureable traces of furfural), and reincubated in distilled water. Controls were treated similarly except that they were re-incubated in furfural. Counts were made as described previously, and the time necessary to effect 50 % activation was determined. These results and the Q_{10} calculated from this data are tabulated in Table 2.

Chemical inhibitors of ascospore germination

During the course of the previous experiments it was observed that many of the compounds that were inactive as activators prevented the germination of heat-activated ascospores. Because of the marked sensitivity of the old spores to poisoning by heterocyclic compounds, these were used in the following experiments with inhibitors. Such old spores showed up to 70 % germination when heat activated but only 15 % when treated with furfural. As a result of these experiments it became apparent that the substitution of a nitro group in the 5-position of an otherwise innocuous heterocyclic compound greatly enhanced its toxicity. A number of such compounds was tested and their toxicity was compared with that of the parent compound, as shown in Table 3.

Table 3. *Effect of the substitution of a nitro-group in the 5-position on the toxicity of furans to germination of ascospores of Neurospora tetrasperma*

(Spores activated by heating at 60° for 30 min.)

Compound	Concentration to cause 50 % inhibition of germination (M)
Furan	$> 1 \times 10^{-3}$ *
2-Nitrofuran	5×10^{-4}
2-Furfuraldoxime	$> 1 \times 10^{-3}$ *
5-Nitro-2-furfuraldoxime	5×10^{-4}
2-Furfurylmethylether	$> 1 \times 10^{-3}$ *
5-Nitro-2-furfurylmethylether	4×10^{-4}
2-Furfurylidine acetone	$> 1 \times 10^{-3}$ *
5-Nitro-2-furfurylidine acetone	5×10^{-3}
2-Acetylpyrrole	$> 1 \times 10^{-3}$ *
5-Nitro-2-acetylpyrrole	4×10^{-4}

* These figures were not defined more precisely because of the insolubility of the compounds.

Table 4. *Heterocyclic compounds which inhibit germination of ascospores of Neurospora tetrasperma*

(Spores activated by heating at 60° for 30 min.)

Compound	Concentration required to cause 50 % inhibition of germination (M)
2-Furoic acid	2×10^{-4}
5-Bromo-2-furoic acid	3×10^{-4}
2-Furyl acrylic acid	5×10^{-4}
2-Pyrrole carboxylic acid	$> 1 \times 10^{-3}$ *
3-Pyrrole carboxylic acid	4×10^{-4}
2-Furyl acrolein	$> 1 \times 10^{-4}$ *
3-Furyl acrolein	8×10^{-4}

* These figures were not defined more precisely because of the insolubility of the compounds.

Table 4 lists the other chemicals found to be toxic to germinating ascospores, and it is at once apparent that heterocyclic carboxylic acids, as a group, are among the most toxic of these chemicals. It should also be noted that the sodium and potassium salts of these acids were relatively non-toxic, as might be expected on the basis of the more ready penetration of the free acid. Finally, experiments were conducted with these substances in which the inhibitors were washed from the spores by immersion in Nacconal followed by several rinses with distilled water. The fact that the washed spores germinated normally demonstrated that the inhibitors used were fungistatic rather than fungicidal.

DISCUSSION

That chemical activators of ascospore germination function in a catalytic role is suggested by the specificity of 5-membered heterocyclic ring compounds in breaking dormancy, and by the small amounts of these compounds required for the activation. On the basis of the preceding experiments, certain generalizations can be made as to the relation between chemical structure and activity among these heterocyclic compounds:

(1) Of the furans and pyrroles tested, only the fully unsaturated compounds were active (tetrahydrofurfuryl alcohol, pyrrolidine and 2:5-dihydropyrrole were inactive in contrast to their completely unsaturated analogues).

(2) The hetero-atom may be either oxygen, nitrogen or sulphur (furans, pyrrole and thiophenes were active).

(3) Conjugated derivatives are not active (haemoglobin, haemin, furoin and furil failed to activate).

The quantitative comparisons given in Table 1 suggest that certain of the furans are the most active, on a molar basis, of the compounds tested. However, it must be remembered that such comparisons are complicated by factors such as permeability, resistance to inactivation by enzymes, etc., so that many secondary characteristics may influence the activity of a compound (Thimann, 1951).

Not much more can be stated positively about the relation between structure and activity than that which is listed above. The active heterocyclic compounds comprise a diverse group of chemical structures, and this fact, together with the observation that diethyl ether is also active, argues against a specific role for heterocyclic compounds in the activation process. Nevertheless, there are certain reasons for believing that such a role does exist. These include: (i) the small amounts required for activation (diethyl ether is required in concentrations as high as 1×10^{-1} M as compared with the heterocyclic compounds effective as low as 1×10^{-4} M); (ii) the toxicity of compounds closely related to activators suggests that inhibition of a key reaction, or reactions, by a structural analogue occurs; (iii) the Q_{10} for furfural activation between 20 and 30° is 4.1, suggesting that a chemical reaction is occurring.

It should also be recalled that some parallels exist between the structural requirements for ascospore activation, and synthetic and naturally occurring higher plant growth regulators. In the latter groups of compounds, several deviations from the main pattern of active structural configuration have also

been observed (Sexton, 1950), but it is still felt that the specificity of certain structures is evidence for their participation in key enzyme reactions (Thimann, 1951). The fact that certain analogues of furfural and pyrrole antagonize the germination process suggests that the use of such specific inhibitors may provide another means of approach to the problem of ascospore activation in *Neurospora* spp.

I wish to thank: Prof. D. R. Goddard who first introduced me to this problem and furnished invaluable help and guidance during my tenure of a National Research Council Fellowship at the University of Pennsylvania during the year 1949-50; the American Cancer Society and the Rackham Fund of the University of Michigan for financial support during some of these investigations; Dr B. O. Dodge for his kindness in sending the cultures of *Neurospora tetrasperma* used in this work; Dr J. Yurchenko of the Eaton Laboratories who furnished the nitrated derivatives used in these experiments.

The co-operation of the research laboratories of the following organizations greatly simplified the course of this work: Cargille Scientific, Inc., New York 6, N.Y.; Commercial Solvents Corp., Terre Haute, Indiana; E. I. Du Pont de Nemours and Co., Inc., Wilmington, Delaware; Eaton Laboratories, Inc., Norwich, New York; Monsanto Chemical Co., St Louis 4, Missouri; The Quaker Oats Company, New York, N.Y.; Reilly Tar and Chemical Corp., Indianapolis, Indiana; F. Ritter and Co., Los Angeles 26, California; Union Carbide and Carbon Corp., New York 17, N.Y.

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(Received 10 July 1952)

SHERATT, H. S. A. & THOMAS, A. J. (1953). *J. gen. Microbiol.* 8, 217-223.

The Nucleic Acid Fractions of a Strain of *Streptococcus faecalis*

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SUMMARY: The Schmidt & Thannhauser (1945) procedure was applied to the nucleic acid fractions of *Streptococcus faecalis*. A part of the deoxypentose nucleic acid was differentiated as being insoluble in *N*-NaOH at 37°, and appeared to be firmly bound to polysaccharide material. Base analyses of the nucleic acid fractions are reported, and qualitative determinations of the amino-acid and sugar composition of the residue described.

The Schmidt & Thannhauser (1945) procedure has been widely used to estimate the nucleic acids in biological materials. It consists, essentially, of incubating the tissue with *N*-NaOH so that the nucleic acids dissolve and the pentose nucleic acid (PNA) is degraded to nucleotides while the deoxypentose nucleic acid (DNA) is still precipitable by acid. The nucleic acid fractions are then resolved by precipitating the DNA, the PNA nucleotides remaining in the supernatant. We have noted, however, in applying this method to a strain of *Streptococcus faecalis*, that a large proportion of the DNA, in association with much cell material, remains undissolved in the *N*-NaOH. Thus the DNA portion appears to exist in two easily separable fractions.

METHODS

Preparation of material. *Streptococcus faecalis* NCIB8123 was grown in a medium comprising 0.2 g. yeast extract (Oxoid), 0.5 g. peptone (Oxoid), 0.5 g. glucose, dissolved in 100 ml. 0.05 M-phosphate buffer and adjusted to pH 7. The organism was grown in 10 l. batches, harvested on a Sharples supercentrifuge, washed twice with water and acetone-dried.

Schmidt & Thannhauser procedure. A modification of this technique (Davidson, Leslie & Waymouth, 1949) was used to prepare the nucleic acid fractions. The dried cells were extracted twice with ice-cold 10 % (w/v) trichloroacetic acid (TCA) in 50 ml. centrifuge tubes, then, in succession, with 80 % (v/v) ethanol, absolute ethanol, chloroform + ethanol (1:3) twice at 80°, and finally with ether.

The remaining dry powder was incubated overnight with 10 ml. *N*-NaOH at 37°. The insoluble residue was centrifuged off and washed with water (15 ml.) which was added to the supernatant. To this combined aqueous solution, 5 ml. of 2.5 *N*-HCl and 6 ml. 30 % (w/v) TCA were added. The DNA was precipitated and spun down by centrifuging for 20 min. at 5000 r.p.m. The precipitate was washed twice with small volumes of 5 % TCA, the washings being added to the supernatant solution which contained the PNA. This

solution of PNA was concentrated *in vacuo*, transferred to a combustion tube and evaporated to dryness in a vacuum desiccator over P_2O_5 and solid NaOH; this was the PNA fraction.

The precipitated DNA was dissolved in a small volume of 0.1 N-NaOH and reprecipitated by acidification with acetic acid and addition of 1 vol. ethanol. The DNA was redissolved in 0.1 N-NaOH and deproteinized by shaking with chloroform + butanol (8:1, v/v). The protein gel was centrifuged off and the DNA precipitated from the aqueous fraction by the addition of acetic acid and ethanol and finally dried with acetone and ether. This was the 'free' DNA fraction.

The insoluble residue from the N-NaOH hydrolysis was washed twice with N-NaOH and then twice with water. It was finally dried with acetone and ether; this was the 'bound' DNA fraction.

The nucleic acids in the three fractions were assayed by direct phosphorus determinations.

Phosphorus determinations. After combustion with 60 % (w/v) perchloric acid, inorganic phosphorus was measured colorimetrically by the method of Fiske & Subbarow (1925).

Nitrogen determinations. Total nitrogen was assayed by a micro-Kjeldahl method.

The Dische reaction for deoxypentoses was carried out qualitatively by heating the sample with two volumes of the Dische reagent (twice recrystallized diphenylamine, 1 g.; sulphuric acid, 1.5 ml.; glacial acetic acid, 100 ml.) for 20 min. at 100°. A blue colour indicates deoxypentose. The specificity of this test was examined by Deriaz, Stacey, Teece & Wiggins (1949).

Chromatographic methods

For nucleic acids the technique of Wyatt (1951) was used. The nucleic acid fractions were hydrolysed by heating with 72 % (w/v) perchloric acid on a boiling water-bath for 90 min., sufficient perchloric acid being used to give an 8 % solution of nucleic acid, calculated from the P content. The hydrolysate was then diluted with 2 vol. water and centrifuged to remove the carbon. Samples (15–25 μ l.) of the supernatant liquid were taken for chromatography and for further P determinations. The chromatograms were run for 36 hr. in isopropanol + HCl (65 : 35, v/v, with 2 N-HCl) on Whatman no. 1 paper and dried in air.

Purines and pyrimidines were detected by photographing the paper in ultraviolet light on reflex document paper. The source of ultraviolet light used was an Osram UV lamp with the outer glass envelope removed. This was in series with a 3000-ohm resistance (mains voltage 230 V. a.c.) and the light was filtered through an Ilford filter no. 828. This is a method which has been used in this laboratory by Mr R. A. Evans. The positions of the spots were traced out on the chromatogram and the areas cut out and eluted, together with the appropriate blank areas, with 0.1 N-HCl. The bases were estimated by measuring their ultraviolet extinction at their maxima against the extinction of the appropriate blank, in the Unicam spectrophotometer. The identity of

all the spots was confirmed by determining the ultraviolet absorption curves of the eluates.

Amino-acids. The sample was hydrolysed with 6 N-HCl for 16 hr., the hydrolysate evaporated to dryness 3 times *in vacuo* and taken up in a small volume of water. The hydrolysates were run on Whatman no. 1 paper with *n*-butanol+acetic acid+water mixture (4:1:5, v/v) in one dimension, and in two dimensions with *n*-butanol+acetic acid+water followed by phenol+NH₃ (3:1 v/v), the NH₃ solution being 3 ml. sp.gr 0.880 ammonia in 1 l. water, using ascending chromatography. The amino-acids were detected by spraying with ninhydrin.

Sugars. The sample was hydrolysed with 2 N-H₂SO₄ in a sealed tube at 100° for 2 hr., the hydrolysate adjusted to pH 4.5 with Ba(OH)₂ solution, and the mixture centrifuged. The precipitated BaSO₄ was washed, and the hydrolysate+washings evaporated to a small volume *in vacuo*. The hydrolysate was run with *n*-butanol+acetic acid+water mixture in one dimension, followed by phenol+NH₃ in the second. The various sugars were detected by aniline hydrogen phthalate (Partridge, 1949). The presence of rhamnose was confirmed by a specific colour test for methylpentoses (Edward & Waldron, 1952).

Growth experiment

One litre of the glucose peptone medium was inoculated with 1 ml. of a 24 hr. culture of *Strep. faecalis* and incubated aerobically at 37°. At hourly intervals 10 ml. samples were withdrawn, centrifuged and the cells washed. The cells were resuspended in 10 ml. water and 1 ml. of this taken and appropriately diluted for turbidity measurements. To the remaining 9 ml., 1 ml. of N-NaOH was added and the suspension incubated at 37° overnight. The residue was then spun down and the ultraviolet absorption of the supernatant read directly in the Unicam spectrophotometer, at its maximum absorption at 260 mμ. This represents the PNA+‘free’ DNA. The residue (‘bound’ DNA) was hydrolysed with 72% perchloric acid at 100° for 1 hr., diluted to 10 ml., the carbon fragments centrifuged down and the ultraviolet absorption of the resulting clear solution measured at its absorption peak of 260 mμ. The absorption curves of the two components were measured and conformed to the normal curve for a nucleic acid, thus showing them to be free from appreciable quantities of other ultraviolet absorbing substances.

RESULTS

The quantitative results are expressed as the means of triplicate determinations on three independent preparations.

‘Bound’ DNA fraction. This is insoluble in N-NaOH at 37°. The cell outlines were still visible and were Gram-negative. The material had P=1.05% and N=3.85% and represented 45% of the cell dry weight. Adenine, guanine, cytosine and thymine were detected and accounted for 78% of the P in the fraction, assuming that the bases in nucleic acids have a 9:10 molar ratio to P (Wyatt, 1951).

The following compounds were also detected in hydrolysates:

(a) Amino-acids: alanine, aspartic acid, glutamic acid, glycine, leucine, lysine, phenylalanine, threonine, tyrosine, valine.

(b) Sugars: galactose, glucose and rhamnose. The fraction gave a strong Dische reaction for deoxypentose.

Extraction of the fraction by boiling *N*-NaOH gradually removed P from the material, although after four successive 15 min. extractions some 10 % of the P still remained in the residue. The P in the supernatants from each successive extraction was found to be almost equally distributed between the acid-soluble and acid-insoluble material. At the same time much of the carbohydrate became soluble in the *N*-NaOH, at a rate corresponding to the extraction of the P. Chromatography showed that purine and pyrimidine bases were also distributed between the acid-soluble and acid-insoluble material.

'Free' DNA fraction. The bases adenine, guanine, cytosine and thymine were detected; they accounted for 95 % of the P of the fraction.

PNA fraction. The bases adenine, guanine, cytosine and uracil were detected; they accounted for 85 % of the P of the fraction. No inorganic P was detected.

Whole cells. Values for the P content of the different nucleic acid fractions were: 'bound' DNA 4.75 $\mu\text{g./mg.}$; 'free' DNA 1.03 $\mu\text{g./mg.}$; PNA 11.6 $\mu\text{g./mg.}$ The purine and pyrimidine composition of the fractions is given in Table 1. The following compounds were detected in hydrolysates of the whole organism:

(a) Bases: adenine, guanine, cytosine, uracil, thymine.

(b) Amino-acids: alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, valine.

(c) Sugars: galactose, glucose, glucosamine, ribose, rhamnose.

Table 1. *The purine and pyrimidine composition of the nucleic acid fractions of washed Streptococcus faecalis*

Base	Molar ratios, calculated to total 4.00		
	'Bound' DNA	'Free' DNA	PNA
Adenine	1.17	1.20	1.00
Guanine	0.73	0.66	1.25
Cytosine	0.81	0.84	0.61
Uracil	—	—	1.14
Thymine	1.29	1.30	—

Results of the growth experiment

The results of the growth experiment are shown in Fig. 1. The amount of bacterial growth was measured by a photo-electric turbidimeter. The relation of this to weight of cell material and to cell numbers once the log phase is reached has been tested by a number of workers (Monod, 1942). The nucleic acid curves were obtained by dividing the ultraviolet absorption by the

turbidity, and thus give a measure of the variation of the amount of nucleic acid/cell during the lag and early log phases, when the mean cell size remains approximately constant. The insoluble fraction persists throughout the growth cycle after the initiation of the log phase and remains proportional to the amount of soluble nucleic acid in the cell. The 'bound' DNA fraction also persists under differing physiological and nutritional conditions (Thomas, unpublished).

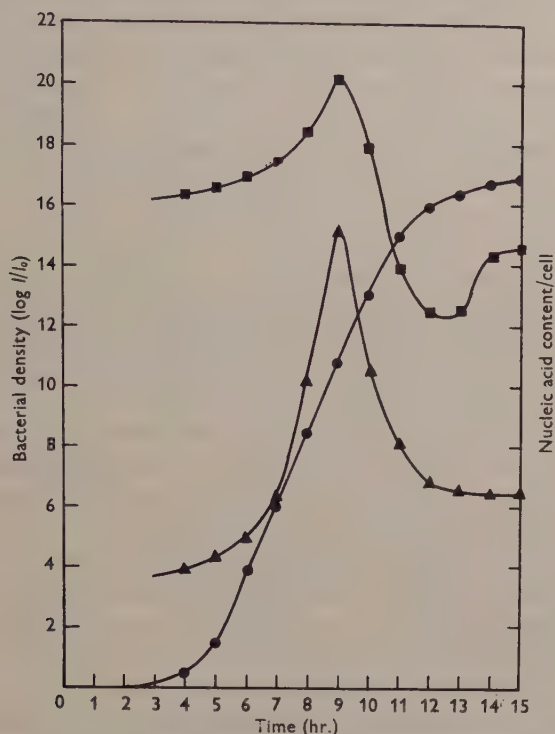


Fig. 1. The variation of the nucleic acid fractions of *Streptococcus faecalis* during growth.

●—● = bacterial density, measured turbidimetrically, $\log I/I_0$; ▲—▲ = 'bound' DNA/cell = ultraviolet absorption at 260 m μ . divided by turbidity; ■—■ = PNA + 'free' DNA/cell = ultraviolet absorption at 260 m μ . divided by turbidity.

DISCUSSION

The drastic conditions necessary for extraction of 'bound' DNA from the cells lead to partial degradation of the DNA to smaller acid-soluble molecules with a simultaneous extraction of carbohydrate. This suggests that the more insoluble DNA fraction is part of a stable complex. If DNA were linked to polysaccharide by ester links between the primary phosphate groups of the DNA and the hydroxyl groups of the sugar residues such a stable complex would be expected. Such esterification of DNA might replace, wholly or in part, electrovalent links to the basic groups of proteins. Purified DNA, extracted from *Haemophilus pertussis*, still contains 33% of carbohydrate (Overend, Stacey, Webb & Ungar, 1951), and carbohydrate is associated

with DNA isolated from rye (Laland, Overend & Webb, 1950). This association of DNA with carbohydrate may be of significance in view of the changes in the serologically active polysaccharide of pneumococci induced by a purified pneumococcal DNA preparation (Avery, MacLeod & McCarty, 1944).

The complex nature of the 'bound' DNA fraction is indicated by the presence of amino-acid and sugar residues. (The finding of purines and pyrimidines, P and deoxypentose is regarded as demonstrating the presence of DNA. This does not, of course, rigidly prove that these components are linked as in DNA). Salton (1952) described the chemical composition of cell walls obtained from *Streptococcus faecalis* by mechanical disintegration. These were largely insoluble in alkali and yielded amino-acids and sugars on hydrolysis. The ultraviolet absorption of the cell-wall suspension gave no evidence for the presence of nucleic acid though the preparation contained 1.8 % P. Our alkali-insoluble residue must contain much somatic material in addition to the cell wall.

There does not seem to be any significant difference in the purine and pyrimidine composition of the two DNA fractions. Both are in the AT (excess adenine and thymine) 'animal' class in contrast to the GC (excess guanine and cytosine) class which Chargaff, Zamenhof, Brawerman & Kerin (1950) have reported for some bacterial DNA preparations.

The chromatographic evidence confirms the validity of the Schmidt & Thannhauser procedure for the separation of the extracted nucleic acids. The finding of ribose in the whole cell is consistent with the presence of a pentose nucleic acid. Most of the P in the 'free' DNA fraction is accounted for by the purines and pyrimidines. The 'bound' DNA has 22 % excess P and the PNA fraction 15 % excess. Mitchell & Moyle (1950) reported that c. 70 % and c. 93 %, respectively, of the P in two strains of *Strep. faecalis* was accounted for by the nucleic acid ultraviolet absorption.

These observations draw attention to the necessity of examining any alkali-insoluble residue, obtained in the Schmidt & Thannhauser procedure, for the presence of nucleic acid.

The authors wish to thank Prof. W. Charles Evans for his interest and encouragement, and Dr W. J. Whelan for helpful advice. We also thank the Agricultural Research Council for their financial support.

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(Received 8 August 1952)

The Occurrence and Distribution of Amino-acid Decarboxylases within the Genus *Lactobacillus*

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SUMMARY: Bacteria possessing active amino-acid decarboxylases, isolated from horse-stomach and sheep-rumen contents, were classified within the genus *Lactobacillus*. One strain, studied in detail, was a homolactic fermenter, the lactic acid formed being optically inactive; lactose was not fermented. Of twenty-six named strains belonging to eight species of the genus, only two possessed amino-acid decarboxylases, namely, one strain of *L. pentoaceticus* and one strain of *L. bifidus*.

The occurrence and distribution of amino-acid decarboxylases among strains of bacteria belonging to many species have been the subject of extensive studies by Gale (1940*a, b*, 1941), but strains of the genus *Lactobacillus* were not included. With the exception of two strains of *L. arabinosus* examined by Eggerth (1939) which did not produce histamine under the conditions of cultivation used, lactobacilli have not been examined for amine production by earlier workers. Of thirty-nine strains of lactobacilli isolated from the human mouth which were examined by Lagerborg & Clapper (1952), a number were shown to possess amino-acid decarboxylases. Two named strains examined, a strain of *L. casei* and one of *L. arabinosus*, showed no decarboxylase activity. The present paper records the examination of strains of *Lactobacillus* spp. isolated from horse stomach and sheep rumen and twenty-six named strains, for amino-acid decarboxylases.

MATERIALS AND METHODS

Growth media

Wheat-mash medium. Wheat-mash medium was prepared by adding 15 ml. of 10 % (w/v) pepsin solution, sterilized by Seitz filtration, to 5 g. sterile ground wheat (autoclaved at 25 lb./sq.in. for 1 hr.). The pH value of the mash was then adjusted to 3.5-4.0 by the aseptic addition of hydrochloric acid.

Wheat-digest histidine broth. One l. distilled water and 100 ml. of 10 % (w/v) pepsin solution were added to 200 g. ground wheat, the pH value adjusted to 3.0 and the mixture incubated at 37° for 4-5 days under toluene, with occasional shaking. The supernatant liquid was collected by decantation and centrifugation, heated to 100°, filtered, and to the filtrate 1 % (w/v) Bacto-peptone (Difco), 1 % (w/v) glucose and 0.1 % (w/v) L-histidine hydrochloride were added. The pH value was adjusted to 6.0 and the medium autoclaved at 10 lb./sq.in. for 20 min. For solid media, 8 % (w/v) Bacto-agar was added before autoclaving.

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Casein liver-digest medium. This contained: casein digest (pancreatic, equivalent to 10 % (w/v) casein), 200 ml.; horse-liver digest (pancreatic, equivalent to 10 % (w/v) liver), 50 ml.; $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$, 10 g.; $(\text{NH}_4)_2\text{SO}_4$, 3 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.01 g.; KH_2PO_4 , 1 g.; K_2HPO_4 , 1 g.; water to 900 ml. The pH value was adjusted to 6.5 and the medium sterilized by autoclaving at 15 lb./sq.in. for 15 min. Glucose (20 g./100 ml. medium) sterilized separately was added after autoclaving. For solid medium, 3 % (w/v) agar and the glucose were added before autoclaving.

Complete, semi-defined medium. The medium used to test the growth factor requirements of the strains had the following composition/10 ml. medium: $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$, 100 mg.; $(\text{NH}_4)_2\text{SO}_4$, 30 mg.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 mg.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.1 mg.; NaCl, 0.1 mg.; KH_2PO_4 , 10 mg.; K_2HPO_4 , 10 mg.; glucose, 100 mg.; casein hydrolysate, 100 mg.; L-tryptophan, 1 mg.; L-cystine, 1 mg.; L-glutamine, 1 mg.; L-asparagine, 1 mg.; thiamine hydrochloride, 1 μg .; pyridoxin hydrochloride, 3 μg .; calcium pantothenate, 1 μg .; riboflavin, 1 μg .; nicotinamide, 1 μg .; biotin, 2 m μg .; *p*-aminobenzoic acid, 3 μg .; pteroylglutamic acid, 0.1 μg .; adenine sulphate, 100 μg .; guanine sulphate, 100 μg .; uracil, 100 μg .; xanthine, 100 μg .; Bactopeptone (charcoal-treated), 3 mg.; Tween 80, 0.001 ml.; choline chloride, 25 μg .; inositol, 25 μg . The pH value was adjusted to 6.5 before autoclaving for 10 min. at 10 lb./sq.in.

Semi-defined medium. This medium, used for the cultivation of strain 30a, had the same composition as the complete semi-defined medium but with the omission of *p*-aminobenzoic acid, guanine sulphate, xanthine, L-glutamine, choline chloride and inositol. In some experiments Bactopeptone was replaced by 0.5 % (v/v) vitamin B₁₂ concentrate prepared from liver ('Examen', Glaxo Laboratories).

Growth temperatures

The incubation temperature for cultures of the unclassified strains of lactobacilli was 37°; cultures of the named species of lactobacilli were incubated at their optimal growth temperature as indicated in Table 3.

Identification of histamine in cultures

Histamine was estimated and identified in cultures of the strains of lactobacilli isolated in Australia by pharmacological assay using the guinea-pig ileum. The neutralized culture supernatants, suitably diluted in Ringer-Tyrode solution, were assayed directly. The specificity of the contractions was checked by the use of drugs (atropine, or the antihistamine drug Neo-antergan: *N'*-*p*-methoxybenzyl-*N'*-pyridyl-*N,N*-dimethylethylenediamine), or by treating the culture supernatants with preparations having diamine-oxidase activity. A paper partition chromatography method was later developed as a qualitative or roughly quantitative method for the identification of histamine in bacterial cultures. Suitable solvent mixtures had the following composition (in parts by volume): (1) *n*-butanol, 100; water, 100; 0.88 sp.gr. ammonium hydroxide, 1; (2) ethyl acetate, 100; water, 100; pyridine, 50. Papers were run in the organic phase of the first solvent mixture

for 4–5 hr., or for 2–3 hr. in the ethyl acetate + pyridine mixture. The papers were then air-dried, and the position of the components giving a positive Pauly diazo reaction determined. The dried papers were sprayed with an aqueous 10 % (w/v) solution of sodium carbonate, again dried in air, and the mixed diazo reagent applied in parallel lines from a fine pipette. The diazo reagent was prepared as described by Koessler & Hanke (1919) except that it was used undiluted.

Determination of decarboxylase activity

The medium used to grow the organisms was casein liver digest medium. The bacterial cells were harvested at the end of the exponential phase of growth, washed once, and resuspended in water. Approximate dry weights of the suspensions were determined by optical density measurement. For the manometric determination of decarboxylase activity the temperature was 30°, oxygen-free nitrogen was used as gas phase and the pH value of the reaction was controlled with McIlvaine buffer.

ISOLATION AND DESCRIPTION OF STRAINS USED

Origin and isolation of strains

The unclassified strains used were isolated in Australia during an investigation of the results of gorging with wheat and other cereal grains in horses and ruminants, a condition ascribed by Åkerblom (1934) to an enterogenous histaminosis, the histamine being formed by bacterial decarboxylation of histidine in the small intestine. Åkerblom implicated an upgrowth of coliform species. In our experiments we found high concentrations of histamine after wheat-feeding, but in contrast to Åkerblom's findings, much higher concentrations were present in the stomach than in the small intestine. In several cases we also found histamine in abnormally high concentrations in the rumen of sheep and goats after wheat-feeding.

Histamine was formed in concentrations as high as 1.7 mg./ml. fluid after incubation of wheat-mash medium inoculated with stomach contents taken from horses fed either on wheat, pasture or hay, or from fasted horses. It was not formed after incubation of uninoculated wheat-mash medium, nor after incubation of mixtures of unsterilized wheat, pepsin and water. The organisms responsible for the formation of histamine in these wheat-mash cultures appeared to be present in the normal microbial flora of the horse stomach.

The microbial flora of the wheat-mash cultures, and also of the stomach of the horse and the rumen of the sheep after wheat-feeding was predominantly Gram-positive, and since coliform species and clostridia were very rare in, or absent from, these materials, it seemed probable that some unrecognized histidine-decarboxylating species were present. Numerous attempts to isolate histidine-decarboxylating organisms by surface culture methods were at first unsuccessful, but success was later obtained with shake cultures. It was subsequently found that the histamine-producing organisms would grow luxuriantly on the surface of suitable solid media in an atmosphere of increased

carbon dioxide tension; after repeated subcultivation they slowly became able to dispense with added carbon dioxide.

After a preliminary enrichment by subcultivation at low pH values in wheat-mash medium, which served to suppress growth of many unwanted species, followed either by shake culture in wheat-digest histidine agar (in which the biotype first isolated grows with a characteristic and easily recognizable appearance, Pl. 1, fig. 1), or by surface culture on a suitable solid

Table 1. *Production of histamine in wheat-digest histidine broth by strains of bacteria isolated from horse stomach and sheep rumen contents*

Cultures were incubated anaerobically in $\frac{1}{2}$ atmosphere of CO₂ for 2 days at 37°. Histamine was estimated by guinea-pig ileum assay.

Strain	Source	Histamine (μ g./ml.)
6a	Horse stomach (pasture fed)	30
6b	Horse stomach (pasture fed)	Nil
30a	Horse stomach (pasture fed)	1200
31a	Horse stomach (pasture fed)	50
2a	Horse stomach (after wheat-feeding)	800
214b	Sheep rumen (after wheat-feeding)	60
214f	Sheep rumen (after wheat-feeding)	Nil

medium incubated in $\frac{1}{20}$ – $\frac{1}{2}$ atmosphere carbon dioxide in hydrogen, a number of histidine-decarboxylating strains were isolated from different sources. Colonies were picked from plate or shake cultures into broth medium, e.g. wheat-digest histidine broth or casein liver digest broth supplemented with histidine (1 mg./ml.), and the formation of histamine in the cultures detected either by guinea-pig gut assay, or on paper chromatograms. By these methods active amino-acid decarboxylating strains were isolated in Australia from stomach contents of three horses on pasture diet, from one horse after wheat-feeding, and from the rumen contents of a sheep after wheat-feeding, and at Cambridge from the stomach contents of a pasture-fed horse.

The concentration of histamine produced after 2 days' incubation in wheat-digest histidine broth by some of the strains isolated in Australia is shown in Table 1. Histamine formation by the strains isolated at Cambridge, as shown by the decrease in intensity of the histidine spot and appearance of a spot in the histamine position on paper chromatograms, is shown in Pl. 1, figs. 2 and 3.

Description of strains isolated

The strains isolated from horse-stomach and sheep-rumen contents have not been identified within the genus *Lactobacillus*. They have been separated into several biotypes on the basis of morphology, cultural appearance, acids produced during growth, and also on the distribution of amino-acid decarboxylases, since Proom & Woiwod (1949) consider the production of amines in culture to be of taxonomic importance.

Biotype 1. Strains of 2a, 30a, 6a and 31a appeared to be closely related or to belong to the same species. Strain 30a has been used in some studies to be reported later, and was examined in more detail. From growth curves

relating dry weight of cell material and period of incubation at temperatures of 30, 35, 40 and 45°, the optimum growth temperature was judged to be 40°.

Strains 6a and 30a were classed as homolactic fermenting organisms. Approximately 90–95 % of the carbon of glucose which disappeared during growth on glucose-containing media appeared as lactic acid. Only traces of volatile acid (identified as acetic acid) were formed. The lactic acid formed by strain 30a was isolated as the zinc salt and was optically inactive.

Table 2. *Nutritional requirements of strains 30a, 2a and 6a*

Medium: complete semi-defined medium lacking each constituent singly. — = no effect on growth; S = growth stimulatory; E = essential nutrient.

Factor	Strain			Factor	Strain		
	30a	2a	6a		30a	2a	6a
	Effect of factor on growth				Effect of factor on growth		
Thiamine	—	—	—	Guanine	—	—	S
Pyridoxin	E	S	S	Xanthine	—	—	—
Pantothenate	E	E	E	Uracil	S	S	S
Nicotinamide	S	S	S	L-Glutamine	—	S	—
Biotin	S	S	S	L-Asparagine	S	—	S
p-Aminobenzoic acid	—	—	S	Tween 80	S	—	S
Pteroylglutamic acid	E	E	S	Bactopeptone	S	S	—
Riboflavin	S	S	E	Choline chloride	—	—	—
Adenine	E	E	E	Inositol	—	—	—

Strains 30a and 6a fermented the same carbohydrates. The carbohydrates fermented by strain 30a were found by its ability to grow in the semi-defined medium to which carbohydrates were added in a concentration of 1 % (w/v). Of the carbohydrates commonly used in systematic bacteriology, glucose, fructose, mannose, sucrose and trehalose supported growth; the greatest yield of cell material was obtained with sucrose. Qualitatively similar results were obtained with strain 6a, for which the amount of growth was judged by titratable acidity.

The nutritional requirements of strains 30a, 2a and 6a were tested by comparing their growth in the complete semi-defined medium with growth in the medium lacking each of the growth-factor constituents singly. The results are summarized in Table 2, in which each nutrient is arbitrarily classed as essential, stimulatory or non-essential. The growth-factor requirements of these three strains were essentially similar. The complete, semi-defined medium did not fully satisfy the nutritional requirements of these strains, since increased growth was obtained by addition of crude liver extracts, or of a refined liver extract containing vitamin B₁₂ ('Examen'). These could not be replaced by purified vitamin B₁₂.

Finally, strains 30a and 2a decarboxylated the same amino-acids. When first isolated, strains 6a and 31a formed large amounts of histamine in culture, but had lost all decarboxylase activity during the interval between their original isolation and testing by the manometric method.

Biotype 2. The strains isolated at Cambridge (C1 and C3) resembled the biotype 1 strains in cultural appearance, but differed in the particular amino-acids which were attacked.

Biotype 3. Strains of 6*b* and 214*f* were unrelated to any of the strains of biotypes 1 or 2. They were not homolactic fermenters, and produced gas during growth in fluid media.

Named strains of the genus *Lactobacillus*. The twenty-six classified strains used to survey the occurrence of amino-acid decarboxylases in named species of the genus are listed in Table 3.

Table 3. *Distribution of amino-acid decarboxylases among strains of lactobacilli*

Strain	Decarboxylation of					
	Arginine	Glutamic acid	Histidine	Lysine	Ornithine	Tyrosine
From horse stomach or sheep rumen. See Table 1	Values of Q_{CO_2}					
30 <i>a</i>	—	—	310	14	20	—
2 <i>a</i>	—	—	200	21	25	—
6 <i>b</i>	8	—	—	—	—	—
214 <i>f</i>	6	—	—	—	—	—
C2.1	44	—	170	—	—	—
C2.3	41	—	125	—	—	—
<i>L. pentoaceticus</i> Rudensis (30° opt.)	26	—	—	—	—	6
<i>L. bifidus</i> ; NCTC4034 (listed as <i>L. acidophilaekogenes</i> Torrey & Rahe; 37° opt.)	21	14	—	—	—	—

Twenty-four other named strains of *Lactobacillus* did not show decarboxylation of the six amino-acids named in this Table. The twenty-four strains were:

Optimum growth temperature 30°.

L. casei strains: W5, NCTC3253, DCEP, YCT1, A094; *L. plantarum* strains: 1-4, 1-8, 4125, NCTC3254; *L. pentoaceticus* strains: 2108; *L. pastorianus* strains: Pasteur, Shimwell, W4, P.S. Lind, P3.

Optimum growth temperature 37°

L. helveticus strains: NCTC4113, CRC2, CRC3, CRCE1, Booth, Happold; *L. acidophilus* strain: TC; *L. delbrueckii* strains: NCTC4033, B.

— = no decarboxylation detected.

The strains labelled NCTC are now kept in the National Collection of Industrial Bacteria, Chemical Research Laboratory (Department of Scientific and Industrial Research), Teddington, Middlesex.

Occurrence of amino-acid decarboxylases in lactobacilli

Washed suspensions of strains 2*a* and 30*a* were examined by the manometric method for their ability to decarboxylate the L-isomers of sixteen amino-acids. The other strains were examined for their ability to decarboxylate the L-isomers of the six amino-acids arginine, glutamic acid, histidine, lysine, ornithine and tyrosine only. The suspensions were tested at pH values

of 4 and 6. The activities for those strains in which amino-acid decarboxylases were detected are recorded in Table 3. The figures shown (expressed as Q_{CO_2}) are the higher of the two values found at the two pH values tested.

DISCUSSION

In the experiments recorded in this paper the optimum conditions for decarboxylase formation, and also the optimum conditions such as pH value and coenzyme concentration, for testing the decarboxylase activities of the washed suspensions, were not determined. The Q_{CO_2} values recorded in Table 3 are probably considerably less than those which might have been found with specially selected conditions. Nevertheless, it is thought that the growth condition of the cells, and the conditions chosen for testing the decarboxylase activities of the washed suspensions, were such that the presence of a particular amino-acid decarboxylase would have been detected if the organism was capable of producing it in significant amounts. The media used supported heavy growth of all the strains examined, equivalent to 0.5–1.5 mg. dry wt. cell material/ml. culture medium. The final pH values of the cultures when the cells were harvested ranged from 4.0 to 5.5 with different strains, and an attempt was always made to harvest the cells towards the end of the exponential phase of growth, when their decarboxylase activities would be expected to be at their greatest (Gale, 1940*a*, *b*, 1941).

The Q_{CO_2} values found for decarboxylation of histidine by washed suspensions of the unclassified strains isolated from horse-stomach contents are greater than those recorded in other species of bacteria. Strains of coliform species had Q_{CO_2} values ranging from 1 to 33 (Gale, 1940*a*), and strains of species of clostridia from 3 to 60 (Gale, 1941). When grown and tested under suitable conditions, it was later found that washed suspensions of cells of strain 30*a* could decarboxylate histidine with Q_{CO_2} values measured at 30° ranging from 500 to 700.

This small survey of the distribution of amino-acid decarboxylases in classified strains of the genus *Lactobacillus* indicates that these enzymes do not occur very widely in classified strains of the genus. It would seem, however, that strains which may be classified within this genus and which do decarboxylate amino-acids very actively, occur quite regularly in such environments as the stomach of the horse. If the possession of these enzymes has a survival value in a strongly acid environment, as first suggested by Hanke & Koessler (1924), an environment such as the stomach of the horse might perhaps favour the selection of those organisms which possess active amino-acid decarboxylating systems, even among such acidophilic bacteria as the lactobacilli.

Strains 30*a* and 6*a* are the only strains isolated which have been studied in sufficient detail to attempt to classify them within the genus. These strains are homofermentative. They do not ferment lactose, which in Bergey's classification would relate them to *L. delbrueckii* or to *L. leishmanii*. The essential difference between these two species lies in their optimal growth temperatures, which is recorded as 45° for *L. delbrueckii* and 36° for *L. leishmanii*.

The optimal growth temperature for strain 30*a* was *c.* 40°, although the rate of growth was slightly higher at 45°. The carbohydrates fermented by *L. delbrueckii* are glucose, fructose, galactose, maltose, sucrose and dextrin, and by *L. leishmanii*, glucose, fructose, maltose, sucrose and trehalose, with slight fermentation of mannitol and α -methyl glucoside (*Bergey's Manual*, 1948). Strains 30*a* and 6*a* fermented glucose, fructose, mannose, sucrose and trehalose. The acid produced by *L. delbrueckii* is recorded as being laevorotatory; that produced by strain 30*a* was found to be optically inactive. Only two authentic strains of *L. delbrueckii* were examined for amino-acid decarboxylases, and none of *L. leishmanii*. Amino-acid decarboxylases were not found in either of the *L. delbrueckii* strains.

The greater part of the work described in this paper was done at Cambridge, while on special study leave from the Division of Animal Health and Production of the Commonwealth Scientific and Industrial Research Organization, Australia. I wish to thank Dr E. F. Gale for his kindness in according me the facilities of the laboratory, and for his constant advice and encouragement. I am grateful to Dr M. Elisabeth Sharpe, of the National Institute for Research in Dairying, Shinfield, Reading, for supplying me with cultures of the twenty-six classified strains of lactobacilli.

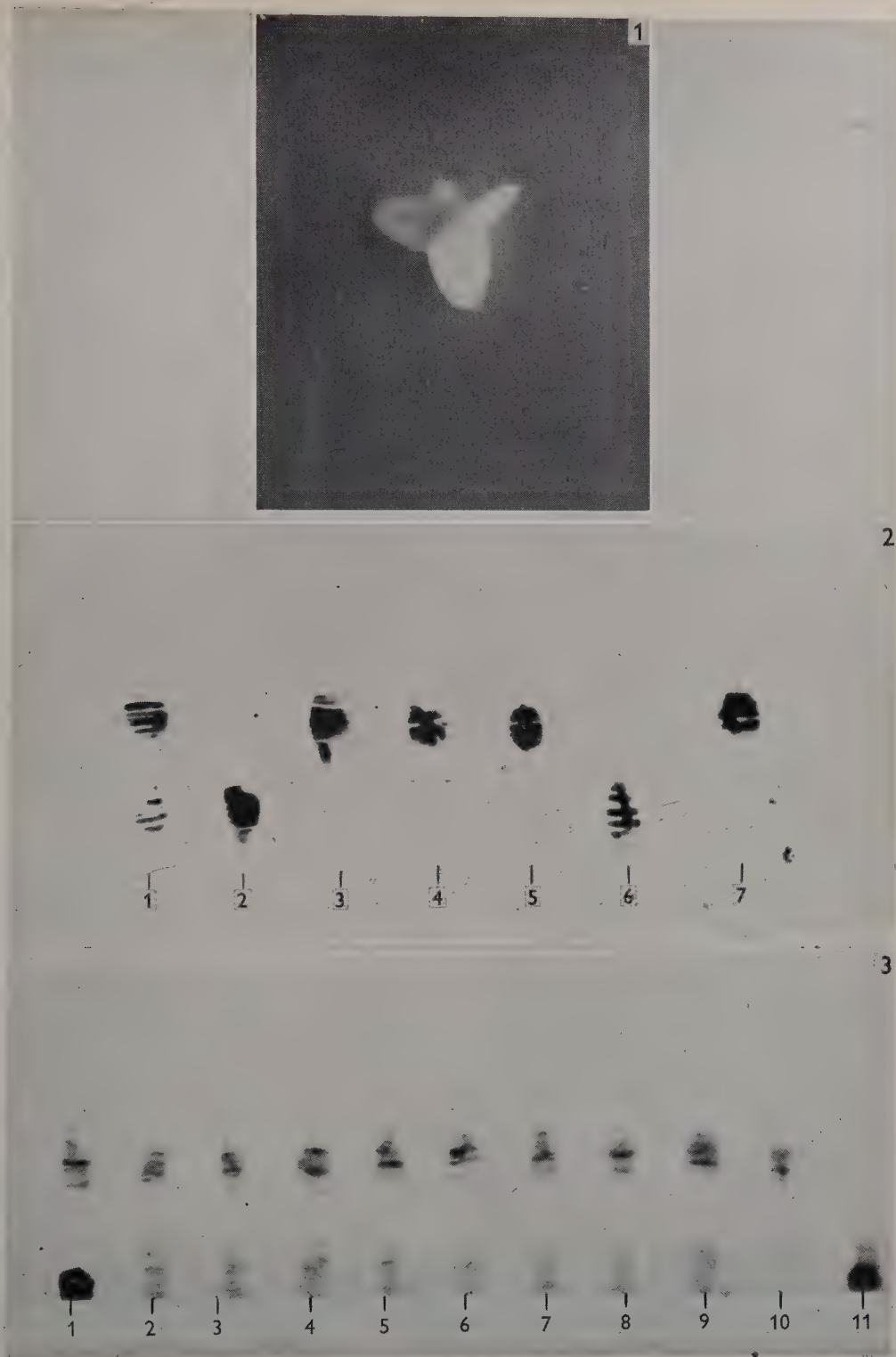
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EXPLANATION OF PLATE

- Fig. 1. *Lactobacillus* strain 6a. Colony from wheat digest histidine agar shake culture after 48 hr. growth at 37°. (Magnification $\times 15$.)
- Fig. 2. Identification of histamine in bacterial cultures by paper chromatography. Paper irrigated $2\frac{1}{2}$ hr. in the organic phase of ethyl acetate + water + pyridine system. R_f histidine 0.07. R_f histamine 0.31. 1, histamine (1 mg./ml.) plus histidine (3 mg./ml.); 2, histidine (3 mg./ml.); 3, histamine (1 mg./ml.); 4-7, culture supernatants, strains 2a-2d.
- Fig. 3. Identification of histamine in bacterial cultures by paper chromatography. Paper irrigated 5 hr. in the organic phase of *n*-butanol + water + ammonia mixture. R_f histidine 0.04. R_f histamine 0.31. 1, histidine (1 mg./ml.) plus histamine (1 mg./ml.); 2-9, culture supernatants of strains 2.1-2.8; 10, histamine (1 mg./ml.); 11, medium control.

(Received 14 August 1952)



A. W. RODWELL—AMINO-ACID DECARBOXYLASES IN *LACTOBACILLUS*. PLATE 1

RODWELL, A. W. (1953). *J. gen. Microbiol.* 8, 233-237.

The Histidine Decarboxylase of a Species of *Lactobacillus*; Apparent Dispensability of Pyridoxal Phosphate as Co-enzyme

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SUMMARY: The histidine decarboxylase produced by a species of *Lactobacillus* (strain 30a isolated from horse-stomach contents; Rodwell, 1953a) has been examined in detail. Attempts to resolve a cell-free enzyme preparation into an inactive apoenzyme and a coenzyme were unsuccessful. Cells grown in a medium deficient in pyridoxin had greatly diminished lysine and ornithine decarboxylase activity, whereas their histidine decarboxylase activity was not at all diminished.

The four bacterial enzymes which specifically decarboxylate the L-isomers of arginine, lysine, ornithine and tyrosine respectively have been shown to require a phosphorylated form of pyridoxal as coenzyme (Gunsalus, Bellamy & Umbreit, 1944; Baddiley & Gale, 1945). The enzymes which attack glutamic acid and histidine have resisted all attempts to resolve them into apoenzyme and coenzyme moieties (Taylor & Gale, 1945; Epps, 1945), and codecarboxylase was not released by boiling histidine and glutamic acid decarboxylase preparations (Epps, 1945). The four enzymes which are known to require pyridoxal phosphate as coenzyme were found to be relatively more sensitive to inhibition by keto (or aldehyde) fixatives such as phenylhydrazine or semicarbazide than were the glutamic acid or histidine decarboxylases (Taylor & Gale, 1945). These findings led Gale (1946) to express the view that the bacterial glutamic acid and histidine decarboxylases do not require pyridoxal phosphate as coenzyme. Umbreit & Gunsalus (1945), however, have described one experiment in which the activity of glutamic acid decarboxylase which was lost after dialysis in the cold at pH 2 was partially restored by the addition of codecarboxylase to the system. Werle & Koch (1949) claim to have shown an activation by pyridoxal phosphate of histidine decarboxylase preparations from both guinea-pig tissue and *Bacterium coli*. Gunsalus (1950) in a review stated that pyridoxal phosphate has now been shown to be the coenzyme for all the amino-acid decarboxylases, whether of bacterial, plant or tissue origin.

However, in the experiments reported in this paper, no evidence was obtained that the histidine decarboxylase produced by a strain of *Lactobacillus* requires codecarboxylase, and it is suggested that more rigid proof is necessary before histidine decarboxylase is included among the enzymes which require pyridoxal phosphate.

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METHODS

Organism and media. The strain of *Lactobacillus* used, strain 30a, and the composition of the semi-defined medium used in the pyridoxin deficiency experiments were described in a previous paper (Rodwell, 1953a).

Determination of decarboxylase activity. This was determined manometrically at a temperature of 30°; the pH value was controlled with McIlvaine buffer and oxygen-free nitrogen was the gas phase. Decarboxylase activities of washed suspensions are expressed as Q_{CO_2} . The notation $Q_{CO_2}(E)$ is used to express the activities of the cell-free enzyme, and is defined as the μ l. CO_2 evolved/hr. by the extract from 1 mg. acetone powder (which corresponds almost exactly to the enzyme obtained from 1 mg. dry wt. of cell suspension).

Preparation of cell-free histidine decarboxylase. The acetone method described by Gale & Epps (1943) for making cell-free preparations of bacterial decarboxylases was found to be very suitable for the preparation of the histidine decarboxylase of strain 30a in the cell-free condition. The optimum pH value for the extraction of enzyme from the acetone-dried powder was from 4.8 to 5.2. Best extraction was obtained by incubating the powder suspended in McIlvaine buffer at pH 4.8 for 8 hr. at 37°. A slightly yellow, sparkling clear extract was obtained after centrifugation. Extract prepared in this way had, in one experiment, an activity expressed as $Q_{CO_2}(E)$ of 480, whereas the activity of the washed suspension used in its preparation had a Q_{CO_2} of 320.

Optimum pH values for the activity of the enzymes. The optimum pH values for the decarboxylation of histidine, ornithine and lysine by washed suspensions were determined in McIlvaine buffer at 4.0, 5.5 and 6.0 respectively. The optimum pH for the cell-free histidine decarboxylase was at pH 4.8.

Preparation of buffer solutions used for dialysis of histidine decarboxylase. Buffer solutions at pH values 2 and 4 were prepared by titrating 0.01M solutions of sodium acetate containing 0.05M-sodium chloride with N-hydrochloric acid by means of a glass electrode; buffer solution at pH 6.0 by titrating 0.01M-citric acid containing 0.05M-sodium chloride with N-sodium hydroxide; and buffer solution at pH 8.6 by titrating 0.01M-boric acid solution containing 0.05M-sodium chloride with N-sodium hydroxide.

Attempts to resolve histidine decarboxylase

Attempts were made to resolve the cell-free histidine decarboxylase prepared from strain 30a by dialysis at 37° against the buffer solutions described above, and also by dialysing the enzyme solution alternately against saturated ammonium sulphate solution made alkaline with ammonia, and against tap water.

Dialysis against dilute buffer solutions. Four portions, each of 10 ml., of the acetone-powder extract were dialysed in cellophane sacs against running tap water for 3 hr., then for 5 hr. in various buffer solutions. The buffer solutions were contained in measuring cylinders (1 l.) standing in a tank of water at 37°, and the buffer solutions (previously warmed to 37°) were changed twice during the 5 hr. period. The extracts were then dialysed against running tap

water for 12 hr. The histidine decarboxylase activity of the undialysed extract, and of the extracts after dialysis, was determined on suitable samples in presence or absence of a boiled, washed suspension of cells of strain 30a; the results are shown in Table 1.

Table 1. *Effect of dialysis against dilute buffer solutions on activity of the cell-free histidine decarboxylase of strain 30a*

Cell-free extracts dialysed against 0.1M buffer solutions at 37°. Activity of dialysed extracts tested in the presence (+) or absence (–) of a boiled aqueous suspension of cells of strain 30a, or of a boiled aqueous acetone powder suspension. Manometer flasks contained extract equivalent to 1 mg. acetone powder and boiled cell or acetone powder extract equivalent to 5 mg. dry wt. Activities are expressed as percentages of the original activity of the undialysed extract, after correction for volume changes during dialysis.

Dialysis at 37° for				
5 hr.				23 hr.
pH value of dialysis				
2.0	4.0	6.0	8.6	8.6
Activity after dialysis as % original activity				
– boiled cells				
6.4	72	80	94	43
+ boiled cells				
6.9	73	83	96	43

The activity of the extracts after dialysis progressively decreased as the pH value of the mixture being dialysed was lowered from 8.6 to 2.0; in no case was a significant reactivation obtained by testing the dialysed extracts in the presence of boiled cell suspension. In another attempt, extract was dialysed against three changes of buffer at pH 8.6 for a total of 23 hr. at 37°. The dialysed extract was tested in the presence and absence of a boiled aqueous suspension of the acetone powder of strain 30a. The activity after dialysis had decreased to 43% of that of the original extract, but no reactivation was obtained by the addition of boiled acetone powder suspension (Table 1).

Dialysis against ammoniacal ammonium sulphate solution. Acetone-powder extract was subjected to the following successive treatments: (i) dialysis against saturated ammonium sulphate solution (containing 3% (v/v) of 0.880 sp. gr. ammonium hydroxide solution) for 2 hr. at room temperature; (ii) dialysis against running tap water for 1 hr.; (iii) dialysis against ammoniacal ammonium sulphate for another 2 hr. period; (iv) against tap water for 12 hr.

The protein precipitated by dialysis against the ammoniacal ammonium sulphate solution was completely redissolved after both subsequent dialyses against tap water. The histidine decarboxylase activity of the dialysed, and of the original undialysed extract, was tested both with and without the addition of a boiled aqueous suspension of cells of strain 30a. The manometer flasks contained extract equivalent to 1 mg. acetone powder, and boiled suspension containing 5 mg. dry wt. of cells. The $Q_{CO_2}(E)$ values found (after correction for volume changes during dialysis) were 440 for the undialysed

suspension both with and without boiled cells, and 286 and 283 for the dialysed extract tested in the presence and absence of boiled suspension respectively.

Effect of a pyridoxin deficiency in the growth medium on the activity of the decarboxylases

Since all attempts to split the histidine decarboxylase of strain 30a into an inactive apoenzyme and a coenzyme were unsuccessful, the deficient culture technique was applied to the problem. Strain 30a has an absolute requirement for pyridoxin (Rodwell, 1953a). The results obtained in two experiments are set out in Table 2. The cells were grown in the semi-defined medium modified by adjusting the pyridoxin content as indicated (Table 2). In both experiments histidine decarboxylase activity was higher in cells harvested from medium deficient in pyridoxin, whereas the ornithine and lysine decarboxylase activities of the cells were very markedly decreased. In these particular experiments only a slight degree of activation of the apoenzymes of ornithine and lysine decarboxylases was obtained by testing pyridoxin-deficient cells in the presence of pyridoxal. However, it was later found that the concentrations of pyridoxal and the time of incubation used in these tests were insufficient to obtain appreciable activation of the apoenzymes in washed cell suspensions of this organism. The activation of ornithine apodecarboxylase in pyridoxin-deficient cells of strain 30a by pyridoxal is described elsewhere (Rodwell, 1953b). The results obtained in one experiment may be anticipated: the rate for the decarboxylation of ornithine by a pyridoxin-deficient cell suspension was $Q_{CO_2} = 5$ in the absence of added pyridoxal, but the rate when the cells were tested in the presence of 0.001 M-pyridoxal increased to a maximum value of $Q_{CO_2} = 1100$. No increase in the rate for the decarboxylation of histidine was ever found when pyridoxin-deficient cells were tested with similar pyridoxal concentrations.

Table 2. *Effect of pyridoxin deficiency in the growth medium on the ornithine, lysine and histidine decarboxylases of Lactobacillus sp. strain 30a*

Cells were grown in semi-defined medium modified by adjusting the pyridoxin content as indicated.

Pyridoxin added to medium ($\mu\text{g./ml.}$)	Decarboxylation of		
	Histidine	Ornithine Q_{CO_2} values	Lysine
10.00	130	116	nt
0.10	232	7	nt
None	185	4	nt
10.00	155	160	220
0.05	264	8	5
None	355	1	2

nt = no test.

DISCUSSION

Failure to resolve the cell-free histidine decarboxylase preparation by the rather severe treatments used may imply a very high affinity of apoenzyme for coenzyme. It would then be expected that a deficiency of pyridoxin in the

growth medium would depress the ornithine and lysine decarboxylase activities of the cells before histidine decarboxylase was affected. The latter might only be affected by extreme deficiency, perhaps at concentrations of pyridoxin too low to allow appreciable growth. In these experiments growth in the medium to which no pyridoxin had been added was approximately half that obtained in the media containing pyridoxin. The production of cells extremely deficient in pyridoxin derivatives by replacing pyridoxin in the growth medium by D-alanine was not tried. These experiments therefore do not prove that the coenzyme for histidine decarboxylase is not pyridoxal phosphate. Nevertheless, when these results are considered together with the earlier work of Epps (1945), and of Taylor & Gale (1945), it is felt that the evidence strongly suggests that histidine decarboxylase does not require pyridoxal phosphate.

I wish to thank Dr E. F. Gale for his interest in this work, which was carried out during special study leave from the Division of Animal Health and Production of the Commonwealth Scientific and Industrial Research Organization, Australia.

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(Received 14 August 1952)

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Factors affecting the Activation of the Ornithine Apodecarboxylase of a Strain of *Lactobacillus*

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SUMMARY: The strain of *Lactobacillus* used (strain 30a) possessed decarboxylases specific for L-histidine, L-lysine and L-ornithine. The rate of decarboxylation increased after a lag period to a maximum value which was dependent on the concentration of pyridoxal. A number of factors was found to affect the rate of activation of the apoenzymes, or, it is thought, probably the rate of pyridoxal phosphate synthesis. Among these factors were: the presence in the test system of the specific substrate for the decarboxylase, the concentration of pyridoxal, and the pH value of the test system. Cells grown in medium deficient in both pyridoxin and folic acid had greatly decreased activation rates for ornithine apodecarboxylase, and pyridoxal phosphate synthesis was also decreased. The addition of folic acid to the system did not affect the rate of activation with folic acid-deficient cells. The addition of thymine to the test system increased the activation rate for folic acid-rich cells, but had no effect with folic acid-deficient cells; other purines were ineffective. It is concluded that some product of thymine metabolism which folic acid-deficient cells are unable to make is concerned with the biological phosphorylation of pyridoxal by this organism.

Bellamy & Gunsalus (1944a) found that cells of *Streptococcus faecalis* grown in pyridoxin-deficient medium contained the apoenzyme of tyrosine decarboxylase. 'Pseudopyridoxine' activated the apoenzyme in living cells, but dried cells prepared from them required adenosine triphosphate (ATP) in addition (Bellamy & Gunsalus, 1944b). With intact cell suspensions and pyridoxal at 1.6×10^{-5} M, complete activation was obtained almost immediately (Bellamy & Gunsalus, 1945). Experiments with the strain 30a of *Lactobacillus* (Rodwell, 1953a) indicated that to obtain cells with high ornithine or lysine decarboxylase activity it was necessary to grow them in media containing concentrations of pyridoxin derivatives very greatly in excess of those required for maximum growth. The ornithine decarboxylase activity of cells grown in such complex media as casein liver-digest medium containing added pyridoxin (10 μ g./ml.) could be further increased in manometric experiments by testing in the presence of high concentrations of pyridoxal.

Ornithine apodecarboxylase was chosen for studies on pyridoxal activation rather than lysine apodecarboxylase because: (a) it appeared to have a very low affinity for coenzyme and required a higher concentration for full activity; (b) it was more stable than lysine apodecarboxylase. With the basal medium they used, Bellamy & Gunsalus (1945) found that apart from pyridoxin,

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nicotinic acid, alanine, purines and pyrimidines, folic acid and related compounds all influenced the amount of complete tyrosine decarboxylase formed without affecting apoenzyme formation.

MATERIALS AND METHODS

Organism. The organism used, *Lactobacillus* sp. strain 30a, has been described previously (Rodwell, 1953a).

Growth media. For the experiments in which the effects of pyridoxal concentration, presence of substrate, and pH value, on the activation of apoenzyme in washed cell suspensions were studied, the medium used for growing the cells was casein liver digest medium. For the experiments in which the effects of a folic acid deficiency were studied, the semi-defined medium (Rodwell, 1953a) was used with the following modifications. The concentration of pyridoxin was decreased to 0.1 $\mu\text{g./ml.}$, which allowed almost maximum mass of growth, although the cells were almost devoid of ornithine and lysine decarboxylase activity (Rodwell, 1953b). For growth of folic acid-deficient cells, folic acid was either omitted altogether or added in concentrations ranging from 0.02 to 0.05 $\text{m}\mu\text{g./ml.}$ For folic acid-saturated cells the medium contained 1 $\mu\text{g.}$ folic acid/ml. The yield of cells from the deficient medium varied from one-quarter to one-half of that obtained in the corresponding folic acid-rich medium. For the experiments in which the effect of the addition of thymine to the growth medium was studied, thymine was added at the rate of 20 $\mu\text{g./ml.}$, together with 0.1 % (v/v) Examen (Glaxo Laboratories Ltd.).

Expression of activities. When testing the ornithine decarboxylase activities of suspensions by activation of the apoenzyme with pyridoxal, the notation Q_{CO_2} (max.) was used to denote the rate/mg. dry wt. cells/hr., calculated from the linear portion of the progress curves. The rate of activation of the apoenzyme was calculated from graphs in which the rate of the reaction for each 5 min. interval was plotted against the reaction time, and is defined as the rate of increase in Q_{CO_2} /hr.

Preparation of ornithine and tyrosine apodecarboxylases. Ornithine apodecarboxylase was prepared from washed cells of strain 30a grown in casein liver digest medium, by extraction of an acetone powder in the same way as for the preparation of histidine decarboxylase (Rodwell, 1953b). During this preparation the ornithine decarboxylase was almost completely dissociated. Tyrosine apodecarboxylase was prepared from cells of *Streptococcus faecalis* (Epps, 1944).

Assay of pyridoxal phosphate. Samples to be assayed were made alkaline with sodium hydroxide, boiled, cooled and neutralized (Green, Leloir & Nocito, 1945). The pyridoxal phosphate content was assayed by means of tyrosine apodecarboxylase, using a standard curve prepared with calcium pyridoxal phosphate (Merck). Acetate buffer was used since Sloane-Stanley (1949) showed that phosphate interferes with the assay.

RESULTS

Pyridoxal activation of ornithine apodecarboxylase in washed suspensions

Effect of pyridoxal concentration. The effect of concentrations of pyridoxal ranging in tenfold steps from 10^{-3} to 10^{-6} M on the progress curves for ornithine decarboxylation is illustrated in Fig. 1. The rate of decarboxylation in the absence of pyridoxal was linear from the beginning of the reaction, whereas

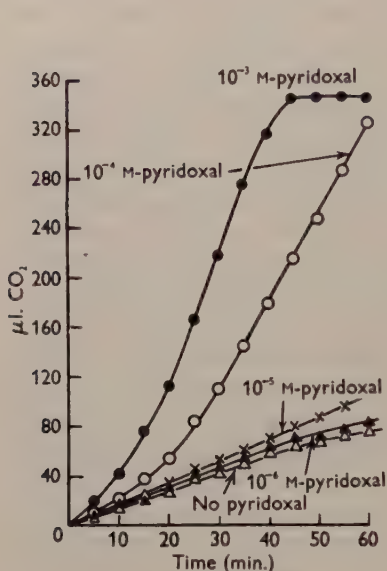


Fig. 1

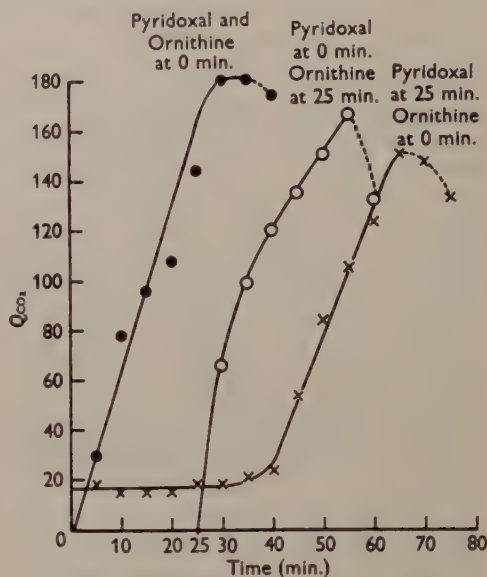


Fig. 2

Fig. 1. Effect of pyridoxal concentration on rate of ornithine decarboxylation by washed cell suspensions. Cells grown in casein liver-digest medium. Manometers contained: 0.2 M-McIlvaine buffer, 1.4 ml.; washed cell suspension (8 mg. dry wt./ml.), 0.5 ml.; pyridoxal hydrochloride solution or water, 0.1 ml.; 0.033 M-ornithine (side-bulb), 0.5 ml.

Fig. 2. Effect of pyridoxal or substrate on the activation rate of ornithine apodecarboxylase in cell suspensions. Manometers contained: 0.2 M-McIlvaine buffer pH 5.4, 1.0 ml.; bacterial suspension (4 mg. dry wt./ml.), 0.5 ml.; 5×10^{-3} M-pyridoxal (side bulb 1), 0.5 ml.; 0.033 M-ornithine (side-bulb 2), 0.5 ml.

in the presence of pyridoxal the rate increased as the reaction proceeded, becoming constant in approximately 30 min. after the addition of substrate in the presence of 10^{-4} M-pyridoxal, and in 20 min with 10^{-3} M-pyridoxal; 10^{-2} M-pyridoxal caused a slight inhibition of the final rate of decarboxylation. In subsequent experiments 10^{-3} M-pyridoxal was used. The rate finally achieved is referred to as $Q_{CO_2}(\text{max.})$, and the rate at which the reaction increases to this maximum value as the rate of activation.

Effect of the presence of substrate on the rate of activation. Ornithine was placed in one side-bulb of a series of double-bulb manometer vessels; pyridoxal solution to give a final concentration after tipping of 10^{-3} M was placed in the

second side-bulb; bacterial suspension and buffer were placed in the main compartments. After equilibrating, ornithine and pyridoxal were added together in one manometer, in the second pyridoxal was added first and ornithine 25 min. later, in the third ornithine was added first and pyridoxal 25 min. later. The rate of activation in the three manometers is illustrated in Fig. 2. Pre-incubation of the cells with pyridoxal, or of cells with substrate, did not greatly alter the rate of activation. It appears that coenzyme is only formed when apoenzyme and substrate are both present; and that apoenzyme, substrate and coenzyme are in dynamic equilibrium. Later experiments in which pyridoxal phosphate assays were performed showed that when the reaction had stopped because of substrate exhaustion, pyridoxal phosphate concentration then decreased. With cell-free ornithine apodecarboxylase and pyridoxal phosphate, a constant rate was obtained immediately after adding substrate.

Effect of pH value on the activation rate and on the Q_{CO_2} (max.). The rates of activation and the Q_{CO_2} (max.) values were determined at a number of pH values ranging from 4 to 6.3; the results are shown in Fig. 3. Whereas the optimum pH value for the activity of the complete enzyme in intact cells was approximately 5.5, the optimum for pyridoxal activation of the apoenzyme was higher than pH 6.3; the optimum for the cell-free ornithine decarboxylase was at pH 5.8.

Relative affinities of ornithine and tyrosine apodecarboxylases for pyridoxal phosphate

The effect of pyridoxal phosphate concentration on the rate of the decarboxylation reaction for a cell-free preparation of ornithine decarboxylase is compared with that for a preparation of tyrosine apodecarboxylase in Fig. 4. The concentrations of pyridoxal phosphate required for half-maximum activity were approximately 1.2×10^{-6} and 2.0×10^{-7} M respectively.

Effect of folic acid deficiency on decarboxylase formation

Relative effects on histidine and ornithine decarboxylases. The effect of a deficiency of folic acid in the growth medium on the ornithine decarboxylase activity of the cells was compared with its effect on histidine decarboxylase activity, and, in one experiment, on the rate of glycolysis. The relative effects of growth in folic acid-deficient medium on histidine decarboxylase, and on ornithine decarboxylase after pyridoxal activation, are recorded in Table 1, in which the activities in each experiment are expressed as percentages of the activities of the cells grown in the corresponding folic acid-rich medium. The results of these experiments indicated that a deficiency of folic acid in the growth medium had a relatively greater effect on the pyridoxal phosphate-requiring ornithine decarboxylase than on histidine decarboxylase, or upon the rate of glycolysis.

Effect of folic acid deficiency on the rate of activation. The rates of pyridoxal activation for folic acid-deficient and acid-rich cells were compared in several experiments. The rates of activation obtained in one experiment are illustrated

Table 1. *Relative effect of growth in folic acid-deficient medium on histidine decarboxylase activity, ornithine decarboxylase activity after pyridoxal activation, and on the rate of glycolysis of the cells.*

The activities are expressed as % of the activities of cells grown in the corresponding folic acid-rich medium.

Exp.	Decarboxylation of		Glycolysis of glucose
	Histidine	Ornithine	
	% activity		
1	98	26	.
2	88	18	.
3	59	24	62

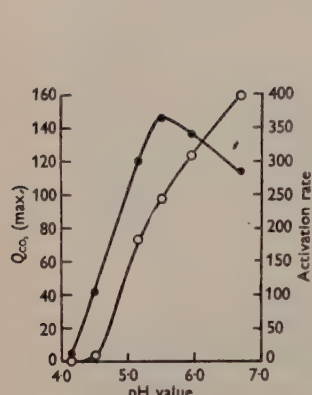


Fig. 3

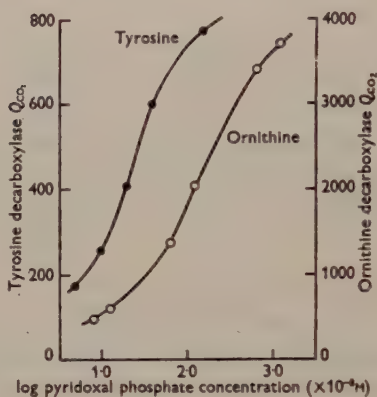


Fig. 4

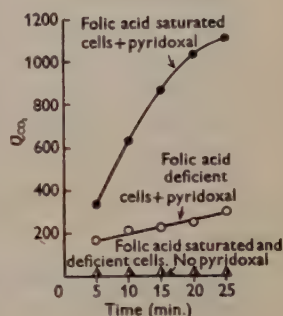


Fig. 5

Fig. 3. Effect of pH value on the rate of activation and on the activity of ornithine decarboxylase in cell suspensions. $\circ-\circ-\circ$ = activation rate; $\bullet-\bullet-\bullet$ = maximum activity. Manometers contained: 0.2M-McIlvaine buffer, 1.4 ml.; bacterial suspension (8 mg./ml. dry wt.), 0.5 ml.; 2.5×10^{-2} M-pyridoxal, 0.1 ml.; 0.033M-ornithine (side-bulb), 0.5 ml.

Fig. 4. Relationship between pyridoxal phosphate concentration and activation of tyrosine and ornithine apodecarboxylases. Manometers contained: (for ornithine) 0.2M-acetate buffer pH 5.6, 1.4 ml.; enzyme solution, 0.1 ml.; pyridoxal phosphate, 1.0 ml.; 0.1M-ornithine (side-bulb), 0.5 ml.; N_2 as gas phase; (for tyrosine) 0.2M-acetate buffer pH 5.5, 1.0 ml.; enzyme solution, 0.25 ml.; pyridoxal phosphate solution, 0.5 ml.; 0.033M-tyrosine suspension (side-bulb), 0.5 ml.; N_2 as gas phase.

Fig. 5. Effect of folic acid in the growth medium on the rate at which cells form the complete ornithine decarboxylase in the presence of pyridoxal. Cells grown in semi-defined medium (pyridoxin concentration 0.1 μ g./ml.; folic acid 1 μ g./ml. for saturated cells, none added for deficient cells). Washed suspensions of cells tested: (a) in the absence of pyridoxal; (b) in the presence of 10^{-3} M-pyridoxal.

in Fig. 5. The activation rate for the folic acid-rich cells was 3800, and for the deficient cells was 400.

Effect of folic acid deficiency on pyridoxal phosphate synthesis. The suspensions were those used to illustrate the effect of folic acid deficiency on the activation rate (Fig. 5). After decarboxylation reaction had proceeded for 25 min., the

manometers were dismantled, sodium hydroxide added to each cup and the contents then boiled and assayed for pyridoxal phosphate. Control mixtures were treated in the same way to obtain the initial pyridoxal phosphate content of the cells. The results of the assays, expressed as $\mu\text{g. pyridoxal phosphate/mg. dry wt. of cells}$, are given in Table 2. The results are in accord with the marked differences in the activation rates of these two suspensions (Fig. 5). In an earlier experiment in which the decarboxylation reaction was allowed to proceed to the point of complete substrate exhaustion, no synthesis of pyridoxal phosphate was found, in agreement with the idea expressed previously that when the reaction has proceeded to completion, the coenzyme may break down again.

Table 2. *Effect of folic acid deficiency on pyridoxal phosphate synthesis in cell suspensions*

Decarboxylation reaction followed manometrically (Fig. 5). Manometers contained: acetate (pH 5.5), 1.5 ml.; bacterial suspension (= 2.5 mg. folic acid-deficient cells, 2.8 mg. saturated cells), 0.25 ml.; 0.1 M-ornithine, 0.5 ml.; 0.01 M-pyridoxal solution, 0.25 ml. After 25 min. reaction manometers dismantled, 0.25 ml. N-NaOH added and contents assayed for pyridoxal phosphate.

	Deficient cells	Saturated cells
	$\mu\text{g. pyridoxal phosphate/mg. dry wt. cells}$	
Initial	0.06	0.06
After 25 min.	0.08	0.34
Synthesis	0.02	0.28

Inhibition of pyridoxal activation by 7-methylpteroylglutamic acid

In several experiments in which it was tried, the rate of activation with folic acid-deficient cells was not increased by the addition of folic acid to the test system; it was necessary that the cells be grown in the presence of folic acid. Inhibition of ornithine decarboxylation by comparatively high concentrations of the analogue 7-methylpteroylglutamic acid was observed. The sensitivity varied with different suspensions, and could be correlated with an inhibition of the pyridoxal activation process rather than with an inhibition of the decarboxylase. The approximate concentrations required for a 20% inhibition of the maximum rate for several suspensions are recorded in Table 3. The same suspension was used for experiments (b) and (c) in Table 3, but it was found that after storage the ability of the suspension to form the complete enzyme from pyridoxal had been largely lost, although there was no deterioration in the activity of the suspension when tested in the absence of pyridoxal. An inhibition of the rate of activation was also observed in these experiments in the presence of the inhibitor.

Effect of thymine on pyridoxal activation

Bellamy & Gunsalus (1945) reported that in addition to folic acid, purines and pyrimidines when added to the basal medium increased the tyrosine decarboxylase activity of *Strep. faecalis* without affecting apoenzyme formation. The semi-defined medium used for cultivating strain 30a contains the purine

adenine, and the pyrimidine uracil. The addition of thymine, but not of guanine, xanthine or hypoxanthine, to medium deficient in folic acid, increased both the rate of pyridoxal activation of the cells, and the Q_{CO_2} (max.) value.

Table 3. *Inhibition of ornithine decarboxylase activity of bacterial suspensions by 7-methylpteroylglutamic acid*

Suspension and growth medium	Concentration of 7MePGA for 20 % inhibition ($\times 10^{-4}$ M)
(a) Cells grown in semi-defined medium (pyridoxin 0.1 μ g./ml.). Suspension inactive in the absence of pyridoxal	3.8
(b) Cells grown in casein liver digest medium. Relatively high activity without pyridoxal, activity increased by pyridoxal	9.5
(c) Same suspension as for (b), but after 24 hr. storage at 4°. Ability to form complete enzyme from pyridoxal lost	19.0
(d) Cells grown in casein liver-extract medium. Ornithine decarb- oxylase saturated with coenzyme. No increase in activity with pyridoxal	47.0
(e) Cell-free enzyme	30.0

(a), (b) and (c) tested in the presence of 10^{-3} M-pyridoxal and inhibitions calculated from Q_{CO_2} (max.) values; (d) tested without activator; (e) tested with excess pyridoxal phosphate.

Table 4. *Effect of thymine on pyridoxal activation of apoenzyme in folic acid deficient and saturated cells*

Manometers contained: acetate buffer (pH 5.5), 1.5 ml.; bacterial suspension (= 1.5 mg. dry wt.), 0.25 ml.; thymine (1 mg./ml.) or water, 0.15 ml.; 0.1 M-ornithine, 0.5 ml.; 0.02 M-pyridoxal, 0.1 ml.

Growth medium (semi-defined + 0.1 % (v/v) Examen) additions	Q_{CO_2} (max.)	
	- thymine	+ thymine
1 μ g./ml. folic acid	780	1100
1 μ g./ml. folic acid	540	490
No folic acid added	280	270

The effect of thymine on pyridoxal activation of the apoenzyme in folic acid-deficient and saturated cells was then studied. The effect of thymine on the maximum rates is recorded in Table 4. Thymine caused an increase in the Q_{CO_2} (max.) with cells grown in the presence of a high concentration of folic acid, whereas folic acid-deficient cells did not respond to thymine. The rate of activation with folic acid-rich cells was also increased by thymine from 1700 to 4800.

Finally, the combined effects of folic acid deficiency and growth in the presence and absence of thymine were studied. The suspensions were in each case tested in the presence and absence of thymine. The Q_{CO_2} (max.) values obtained are recorded in Table 5. In this experiment also the maximum activity of cells grown in medium deficient in folic acid, and lacking thymine, was not increased by testing them in the presence of thymine.

Table 5. *Effect of thymine on pyridoxal activation in cells grown in medium deficient and rich in folic acid, and with and without the addition of thymine*

Manometers contained: acetate buffer (pH 5.5), 1.4 ml.; bacterial suspension (=1.2 to 1.4 mg./ml. dry wt.), 0.25 ml.; thymine (1 mg./ml.), 0.1 ml.; 0.01 M-pyridoxal, 0.25 ml.; 0.1 M-ornithine, 0.5 ml.

Additions/ml. medium (semi-defined medium + 0.1 % (v/v) Examen)		Q_{CO_2} (max.)	
Folic acid	Thymine	- Thymine	+ Thymine
1 μ g.	—	1000	1240
1 μ g.	20 μ g.	1140	1280
0.1 m μ g.	—	660	640
0.1 m μ g.	20 μ g.	900	1100

DISCUSSION

The concentration of pyridoxal required to obtain maximum ornithine decarboxylase activity with washed suspensions of cells of the strain 30a lactobacillus, is greater than that required for full activation of tyrosine apodecarboxylase with cells of *Streptococcus faecalis*; nor is there a delay with cells of the latter organism before full activity is reached (Bellamy & Gunsalus, 1945). Possible explanations for these differences are: (a) pyridoxal may only enter cells of strain 30a at a comparatively slow rate; (b) strain 30a may have a relatively inefficient pyridoxal phosphorylating system; (c) ornithine apodecarboxylase may have a relatively low affinity for the coenzyme, and a high concentration must be maintained by the cell to saturate the apoenzyme. The concentrations of pyridoxal phosphate required to obtain half maximum activity for tyrosine and ornithine apodecarboxylases were found to be 2.0×10^{-7} and 1.2×10^{-6} M respectively.

It has been suggested that apoenzyme, substrate and pyridoxal must all be present before coenzyme synthesis occurs. If this be true, the effects of folic acid deficiency on the activation rates, pyridoxal phosphate synthesis, and on the Q_{CO_2} (max.) values, could all be explained by a decreased formation of apoenzyme in cells grown in folic acid-deficient medium. The effect of folic acid deficiency on apoenzyme formation was not investigated. There seems little reason to suppose, however, that the formation of the apoenzyme of ornithine decarboxylase would be affected by folic acid deficiency to a greater degree than histidine decarboxylase, or than the constitutive enzymes concerned with glycolysis. The experiments with 7-methylpteroylglutamic acid also support the view that cells grown in medium deficient in folic acid have a decreased ability to form coenzyme from pyridoxal. Martin & Beiler (1947) found that the 3:4-dihydroxyphenylalanine (dopa) decarboxylase of rat kidney was inhibited by 7-methylpteroylglutamic acid, and that the inhibition was annulled by folic acid; tyrosine decarboxylase of *Strep. faecalis* was much less sensitive under the same conditions. They inferred from this evidence that folic acid forms part of the dopa decarboxylase system. It would appear

likely that in the experiments of Martin & Beiler folic acid was concerned with the maintenance of pyridoxal phosphate concentration; Green *et al.* (1945) had previously shown that dopa decarboxylase requires pyridoxal phosphate.

Bellamy & Gunsalus (1945) reported that the presence of purines and pyrimidines, as well as folic acid, in the growth medium, all influenced the formation of the holoenzyme of tyrosine decarboxylase in cells of *Strep. faecalis* without affecting apoenzyme formation. In the present studies, the inclusion of thymine in the growth medium increased the Q_{CO_2} (max.) values of the cells grown in it, and thymine also had an effect on the pyridoxal activation system in washed suspensions. It increased both the rate and the Q_{CO_2} (max.) value of suspensions of cells grown in folic acid-rich media, but was without effect with cells grown in media poor in folic acid and lacking this pyrimidine. It seems probable, therefore, that some product of thymine metabolism, which washed suspensions of folic acid-deficient cells are unable to make, is concerned with the uptake or phosphorylation of pyridoxal by this organism. There is evidence that both the active form of folic acid and vitamin B₁₂ are involved in the conversion of thymine to thymidine and to other deoxyribosides (Shive, Eakin, Harding, Ravel & Sutherland, 1948; Franklin, Stokstad, Hoffman, Belt & Jukes, 1949; Jukes, Broquist & Stokstad, 1950).

I wish to thank Dr E. E. Gale for his encouragement and advice. I am indebted to the Medical Research Council of Great Britain for a generous grant during the period of this work, which was carried out during special study leave from the Division of Animal Health and Production of the Commonwealth Scientific and Industrial Research Organization, Australia.

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(Received 14 August 1952)

The Steroid Requirements of *Labyrinthula vitellina* var. *pacifica*

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SUMMARY: *Labyrinthula vitellina* var. *pacifica* requires a steroid as growth factor. Cholesterol, Δ^4 -cholestenone, fucosterol, and β -sitosterol are active. When cholesterol is modified by (1) the C3 \ddagger hydroxyl group becoming *trans* to the C10 methyl group, (2) esterification of the C3 hydroxyl group, or (3) saturation of the C5, 6 double bond, the resulting compound is inactive. Modification of the side chain of cholesterol or cholestenone by the addition of a C24 ethyl or vinyl group does not destroy activity but unsaturation at C22, 23 does, as do the more marked changes found in diosgenin, deoxycorticosterone acetate, and methyltestosterone. Bile salts are inactive. The specificity of the requirement indicates that active steroids function as essential metabolites rather than solely as protective agents. Active steroids may be obtained by this micro-organism in nature from host plants or from diatoms and bacteria associated with the hosts.

The isolation in pure culture of the labyrinthula strains by Watson (to be published) made possible the investigation of their nutritional requirements. One isolate, *L. vitellina* var. *pacifica*, has been found to require a steroid for growth. Since such requirements are rare among micro-organisms, a detailed investigation of steroid specificity has been made.

MATERIALS AND METHODS

Stock cultures of isolates of *L. macrocystis* var. *atlantica* and *L. minuta* var. *atlantica* were maintained at 20° in tubes containing the semi-solid medium given in Table 1. The addition of 1.0 mg % (w/v) of cholesterol rendered this medium suitable for the maintenance of *L. vitellina* var. *pacifica*.

The agar in this medium appears to be necessary as a physical support without which these organisms settle to the bottom of the tube, or cling to its sides, where they grow poorly. High viscosity methylcellulose (0.5 % (w/v), Hercules Powder Company) gave fair results as an alternative supporting agent but did not form a sufficiently homogeneous gel to give consistent or maximal growth. The constant motion provided by a rotating apparatus constructed after Baker (1949) substituted for mechanical support and allowed the smallest strain (*L. minuta* var. *atlantica*) to grow as well in a liquid as in an agar-containing medium. Tubes of liquid medium were placed in a rack held at an angle of 11.5° to the horizontal and rotated at 21 r.p.m. by this apparatus. This method of cultivation appears most promising for more critical studies

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‡ For the structure of steroids and the numbering system used see Deuel (1951).

of non-steroid nutritional requirements of *labyrinthula* and is being further studied.

Since it was most convenient to use agar as supporting agent for the present investigations, only preliminary experiments on non-steroid nutritional requirements have been made. The agar used (Difco agar) contains significant amounts of various vitamins and inorganic compounds. The latter are also contributed

Table 1. *Composition of stock medium. Quantities made up to 100 ml. with distilled water and pH value adjusted to 8.0–8.2 with KOH after the medium had been boiled for 20 min. and cooled to room temperature*

NaCl	2.5 g.	Thiamine	0.2 mg.
MgSO ₄ .7H ₂ O	0.5 g.	Nicotinic acid	0.1 mg.
K ₂ HPO ₄	0.01 g.	Ca pantothenate	0.1 mg.
Ethylenediamine tetracetic acid*	0.05 g.	Pyridoxin.HCl	0.04 mg.
CaCO ₃	0.025 g.	Pyridoxamin.2HCl	0.02 mg.
Mn (as manganous sulphate)	2.0 mg.	p-Aminobenzoic acid	0.01 mg.
Zn (as sulphate)	2.0 mg.	Biotin	0.5 µg.
Fe (as ferrous sulphate)	0.2 mg.	Cobalamin (vitamin B ₁₂)	0.05 µg.
Gelatin hydrolysate	0.1 g.	Folic acid	2.5 µg.
Agar	0.2 g.		

* 'Versene'-free acid, Bersworth Chemical Co., Framingham, Mass.

to the medium by the gelatin hydrolysate. The gelatin hydrolysate used was made from Eastman gelatin hydrolysed with sulphuric acid and partially neutralized with barium hydroxide before filtration and final neutralization with KOH. The strains which we have maintained on the stock medium (Table 1) grow only slightly better and more consistently when the vitamins listed are added than when they are omitted. It seems probable that these strains do require one or more of the known vitamins. Our preliminary experiments indicated that the sodium, chloride, phosphate, magnesium, calcium, manganese, zinc and iron contents of the stock medium are approximately optimal.

Gelatin hydrolysate serves as carbon and nitrogen source. Glucose, sodium succinate hexahydrate, fumaric acid (neutralized with KOH), glycerol, and sodium acetate trihydrate were ineffective as substrates in the presence of a non-toxic concentration of NH₄Cl (0.02 %, w/v) added aseptically after autoclaving. The carbon sources tested were at the concentration of 0.2 % (w/v). Monosodium glutamate (0.25 %, w/v) supported a slight amount of growth. The initial pH value of the medium could not be lowered without decreasing growth.

Under the conditions given stock cultures should be transferred once a week, although they will survive for a month if kept in the refrigerator after a 4- or 5-day period of growth at 20°. The temperature tolerance of *L. minuta* var. *atlantica* extends from about 2° (refrigerator) to 30°; cultures incubated at 37° failed to grow at that temperature or when transferred to a room temperature of about 21–24°. *L. vitellina* var. *pacifica* and *L. macrocystis* var. *atlantica* grew rapidly at 20° but were killed by incubation at 30–31°.

RESULTS

L. vitellina var. *pacifica* was originally isolated on serum agar and did not grow in the absence of serum. The discovery that cream, but not yeast extract or a tryptic digest of casein, would substitute for serum suggested that a lipid growth factor was required. Non-toxic concentrations of bile salts (1.0, 5.0 mg./100 ml.), lecithin (1.0, 5.0, 10.0 mg./100 ml.), Tween 40 (1.0 mg./100 ml.; polyoxyethylene sorbitan palmitate, Atlas Powder Co.), Tween 80 (1.0 mg./100 ml.; polyoxyethylene sorbitan oleate, Atlas Powder Co.), or glycerol mono-oleate (1.0 mg./100 ml.) failed to support continued growth. Cholesterol (1.0 mg./100 ml.) was effective in preliminary experiments.

The stock medium (without cholesterol) was used in studying the specificity of this steroid requirement. Ten ml. lots of medium were distributed in 25 ml. glass-capped Erlenmeyer flasks which were in turn sealed with transparent cellulose tape between two Pyrex glass kitchen trays. Steroids were added as freshly made solutions in commercial absolute ethanol, before autoclaving. Ergosterol was also used as an unsterilized ethanolic solution added after autoclaving. No contaminations occurred in this experiment. The amount of ethanol added is critical; 0.1 ml. ethanol/10 ml. medium added after autoclaving completely suppressed growth in the presence of cholesterol. Ethanol is not completely removed by autoclaving; 0.25 ml. ethanol/10 ml. medium added before autoclaving inhibited growth although half this amount did not. No more than 0.05 ml. ethanol/10 ml. medium was added after autoclaving, nor more than 0.125 ml./10 ml. before autoclaving, in these experiments. The addition of the steroids as ethanolic solutions gave fairly uniform dispersion. No better results were obtained by using bile salts to solubilize cholesterol. The inoculum consisted of one or two drops of a 1/10 dilution of a stock culture in basal medium. Inoculated flasks were incubated in an incubator controlled at 18–20°.

The steroid preparations used were: cholesterol (U.S.P.); cholesteryl acetate (recrystallized from a preparation of Dr N. A. Milas); cholesteryl laurate (m.p. 74–75°, gift of Dr M. Calvin, Radiation Laboratory, University of California); cholesteryl palmitate, *epicholesterol*, progesterone, estrone, and deoxycorticosterone acetate (gifts of Dr H. Sobotka, Mt. Sinai Hospital); coprosterol (m.p. 92–99°), β -cholestanol (m.p. 145°), Δ^4 -cholestenone (m.p. 79–80°), and cholestanone (m.p. 129–130°) (gifts of Dr Ivan Salamon, Sloan Kettering Institute for Cancer Research); ergosterol (The Matheson Co., Inc., East Rutherford, N.J., recrystallized before use); ergosterol, zymosterol (m.p. 108–115°, $[\alpha]_D + 34$ [CHCl₃]), and 5-dihydroergosterol (gifts of Dr O. N. Breivik, the Fleischmann Laboratories); fucosterol, brassicasterol, and β -sitosterol (gifts of Dr W. Bergman, Sterling Chemistry Laboratories, Yale University); β -sitosterol (L. Light and Co., Ltd., Colnbrook, Bucks, England); mixed soya sitosterols (β and γ) and stigmasterol (The Glidden Co., Chicago, Ill.); calciferol (Nutritional Biochemicals Corp., Cleveland, Ohio); methyltestosterone and ethinyl estradiol (Schering Corp., Bloomfield, N.J.); cortisone (gift of Dr T. H. Jukes, Lederle Laboratories); diosgenin (gift of Dr Monroe

E. Wall, Eastern Regional Research Laboratory, United States Department of Agriculture); and progesterone (gift of Dr K. Schindler).

Table 2. *Steroids as growth factors for Labyrinthula vitellina var. pacifica*

	Steroid added to 10 ml. basal medium	Optical density
None; no inoculum	0	0.07
Cholesterol	0.05 mg.	0.07
None; inoculated	0	0.07, 0.08
Cholesterol	0.005 mg.	0.09*
	0.01 mg.	0.17-18
	0.025 mg.	0.31
	0.05 mg.	0.31, 0.36, 0.41
	0.1 mg.	0.37
	0.25 mg.	0.41
	0.5 mg.	0.50
Δ^4 -Cholestenone	0.0025 mg.	0.09*
	0.005 mg.	0.19, 0.12†
	0.01 mg.	0.30
	0.025 mg.	0.33
	0.05 mg.	0.37
	0.1 mg.	0.37, 0.41
	0.25 mg.	0.33
Fucoesterol	0.005 mg.	0.12-13†
	0.01 mg.	0.25
	0.025 mg.	0.31
	0.05 mg.	0.33
β -Sitosterol	0.005 mg.	0.08*
	0.01 mg.	0.16
	0.025 mg.	0.30
	0.05 mg.	0.34-36, 0.29
	0.1 mg.	0.32-33
	0.25 mg.	0.38-40

* Indicates absence of growth, † slight growth (150 organisms or more in a low power microscope field) where the optical density data do not clearly provide this information.

The positive results are given in Table 2 in terms of the optical density of the cultures reached after 8 days of incubation. Cholesterol, Δ^4 -cholestenone, fucoesterol and β -sitosterol were active as growth factors. The following compounds showed slight activity at the concentrations given parenthetically: *epicholesterol* (0.1 mg./10 ml.), *so yastosterols* (0.025 mg./10 ml.; higher concentrations toxic), *ergosterol* (added after autoclaving, 0.01-0.1 mg./10 ml. but not consistently), *brassicasterol* (0.01-0.1 mg./10 ml.), and *stigmasterol* (0.1 mg./10 ml.). We consider these compounds to be really inactive because of the low level of growth (optical densities usually less than 0.19) and the relatively high concentrations required for even this little growth. The purity of these steroid preparations was not high and steroids deteriorate more or less rapidly in any case; contaminants or degradation products were probably responsible for the slight apparent activity. When the occurrence of growth was doubtful a microscopic examination of the culture was always made. The

Other compounds tested were inactive, although not completely inhibitory over at least a portion of the experimental range of concentrations. They were as follows: cholesteryl acetate (toxic at 0.1 mg./10 ml.), cholesteryl laurate and cholesteryl palmitate (toxic at 0.025 mg./10 ml.), 3- β -cholestanol (inhibitory at 0.1 mg./10 ml.), coprosterol (toxic at 0.05 mg./10 ml.), cholestanone (inhibitory at 0.1 mg./10 ml.), 5-dihydroergosterol, zymosterol (toxic at 0.05 mg./10 ml.), calciferol (toxic at 0.025 mg./10 ml.), cortisone, deoxycorticosterone acetate (toxic at 0.05 mg./10 ml.), estrone (toxic at 0.01 mg./10 ml.), ethinyl estradiol (toxic at 0.01 mg./10 ml.), methyltestosterone (inhibitory at 0.025 mg./10 ml.) and diosgenin. Progesterone was so toxic that no growth occurred at the lowest concentration tested—0.005 mg./10 ml. of medium. A compound was judged to be toxic when it completely inhibited growth in the presence of 0.05 mg. cholesterol/10 ml. medium. The significance of the apparent inactivity of the more toxic compounds is doubtful for the same reasons that cause us to doubt the significance of slight activities.

Comparison of the structures of active and inactive compounds leads to the following conclusions:

(1) The oxidation of the hydroxyl group of C3 to a keto group, and the simultaneous shift of the double bond from 5:6 to 4:5 does not affect activity since cholestenone was active.

(2) The configuration of the hydroxyl group at C3 must be *cis* to the C10 methyl group; *epi*cholesterol was inactive.

(3) Esterification of the hydroxyl group destroys activity; none of the cholesteryl esters was active.

(4) Saturation of the 5:6 double bond destroys activity; neither coprosterol nor its C5 stereoisomer, cholestanol, were active. Saturation of the 4:5 double bond of cholestenone likewise destroys activity; cholestanone was inactive.

(5) The side chain of cholesterol may be modified by the addition of an ethyl group at C24, but only one of the two possible C24 stereoisomers resulting is active; the activity of mixed β - and γ -sitosterols occurred at a level indicating activity of β -sitosterol alone.

(6) A vinyl group at C24 is also permissible; fucosterol was active.

(7) Unsaturation of the side chain at C22, 23 destroys activity; stigmasterol was inactive although β -sitosterol (22-dihydrostigmasterol) was active.

(8) Changing the side chain of cholesterol to that of a saponin destroys activity; diosgenin was inactive.

(9) Shortening the side chain of cholestenone to that of deoxycorticosterone acetate or methyltestosterone destroys activity. The other compounds tested differed in more than one respect from active compounds.

DISCUSSION

The physiologic importance of steroids in higher animals is well recognized. However, while steroids are found in many groups of micro-organisms (Deuel, 1951), they do not occur in all micro-organisms. Sifferd & Anderson (1936) isolated significant amounts of a steroid mixture from *Azotobacter chroococcum* which had been grown on a steroid-free medium; Guirard, Snell & Williams

(1946) isolated a steroid fraction from several *Lactobacillus* spp. Yet Anderson, Schoenheimer, Crowder & Stodola (1935), applying even more searching techniques to *Mycobacterium tuberculosis*, were able to confirm the many earlier reports which stated that no steroids could be found in these bacteria. *Corynebacterium diphtheriae* (Chargaff, 1933*b*), the BCG strain of *Mycobacterium tuberculosis* (Chargaff, 1933*a*), and *Bacterium coli* (Behring, 1930) also appear to lack steroids. Heilbron (1942), reviewing the work of his laboratory on the steroid composition of algae, reported that the blue-green algae contained no steroids. It is therefore difficult to postulate a general essential role of steroids in micro-organisms.

Evidence that steroids may nevertheless be essential metabolites for a few micro-organisms has been provided by nutritional experiments. The degree to which such requirements represent a special metabolic evolution will become clearer when more such organisms are known, and when we have some idea of the biochemical role of these compounds. Unfortunately, the demonstration that cholesterol is a necessary ingredient in a medium does not prove that it has a metabolic role within the micro-organism. The toxicity of unsaturated fatty acids was first noted by Hutner (1942) to be annulled by cholesterol, among other compounds. This subject has been discussed in detail recently by Pollock (1949) and by Kodicek (1949). Ordinary bacteriological broths may require the addition of cholesterol solely as a protective agent for sensitive organisms (see Lwoff 1947, on the subject of peptone media for *Moraxella* and *Neisseria*). A medium initially fatty acid-free can be made toxic by unsaturated fatty acids produced by a micro-organism before visible growth has occurred (as *Haemophilus pertussis*, see Pollock, 1949). Organisms which require oleic acid as a growth factor may therefore require cholesterol, or other substances with a similar 'buffer' action, to maintain a low but constant supply of oleic acid. It follows that pure cultures, media of known composition, and knowledge of the specificity of the requirement, are necessary if one is to conclude for a given micro-organism that a steroid is an essential metabolite rather than solely a protective agent.

None of these desiderata has yet been attained in the nutritional studies which indicate cholesterol requirements in amoebae (see Lwoff, 1951). It is difficult to evaluate Devloo's (1938) claim that yeast 'W' required 'biosterol' in the light of present-day steroid chemistry. The specificity studies of Cailleau (1937) on the steroid requirement of a pure culture of *Trichomonas columbae*, however, indicate a true metabolic requirement which may be general in this group of protozoa. *Eutrichomastix colubrorum* (Cailleau, 1938*b*) in pure culture, and *Trichomonas batrachorum* (Cailleau, 1939) and *T. foetus* (Cailleau, 1938*a*) in mixed cultures also required cholesterol. Unpublished data of Hutner and Sanders have confirmed the cholesterol requirement of *T. foetus* in pure culture. The only other steroid-requiring micro-organisms which have been reported are the pleuropneumonia-like organisms studied by Edward & Fitzgerald (1951), which required cholesterol; of the related compounds tested stigmasterol and cholestanol were active.

The metabolic role of cholesterol in these organisms, and in labyrinthula,

is unknown. The specificity of the requirement rules out the possibilities suggested by the activity of cortisone (Gaines, Broquist & Williams, 1951) and dehydroepiandrosterone (Gaines & Totter, 1950) in replacing leucovorin and pteroylglutamic acid for *Leuconostoc citrovorum* and *Streptococcus faecalis*. Furthermore, folic acid was present in our basal medium for labyrinthula. It is also interesting to note that the specificity of the steroid requirement of *L. vitellina* var. *pacifica* differs from that of *Trichomonas columbae*. *T. columbae* utilized 5:6 saturated sterols, cholesteryl esters and ergosterol as growth factors; labyrinthula did not. Conversion of the C3 hydroxyl group to a keto group destroyed activity for *Trichomonas columbae* but not for labyrinthula. The pleuropneumonia-like organisms of Edward & Fitzgerald utilized cholesterol and stigmaterol, but not cholesteryl esters, ergosterol, or coprosterol, thus exhibiting still another pattern of specificity.

Where does *L. vitellina* var. *pacifica* obtain steroids in nature? This variety was found associated with *Zostera marina*, *Ulva* spp., and material from phytoplankton and zooplankton tows. The common steroids of higher plants (as *Zostera marina*) are the sitosterols and stigmaterol; of these β -sitosterol is active. 'Sitosterol' is also commonly present in the green algae (Heilbron, 1942), including *Ulva lactuca*. Diatoms and bacteria are conspicuous associates of the host plants. The diatom *Nitzschia closterium* contains fucosterol (Heilbron, 1942) which is active. Evidence that bacteria may provide utilizable steroids (though labyrinthula is not phagotrophic) was presented by isolation plates of non-nutrient sea-water agar on which *L. vitellina* var. *pacifica* grew in and around bacterial colonies but not when more distant from bacteria.

We are very grateful to those (indicated in the text) whose generosity in donating steroids made this study possible. We also wish to thank Michael Bach for assistance and Dr S. H. Hutner and Dr W. Vishniac for discussion of this work. The Haskins Laboratories investigations of microbial nutrition are assisted by grants from the Lederle Laboratories Division of the American Cyanamid Co. and the Rockefeller Foundation.

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(Received 18 August 1952)

EDWARD, D. G. FF. (1953). *J. gen. Microbiol.* 8, 256-262.

A Difference in Growth Requirements between Bacteria in the L-phase and Organisms of the Pleuropneumonia Group

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SUMMARY: Serum could be replaced in media for growing the L-phase of *Proteus vulgaris* by a phospholipid fraction from egg yolk and for growing the L-phase of *Streptobacillus moniliformis* by the addition of this fraction + bovine albumin. Organisms of the pleuropneumonia group grew in media in which serum was replaced by these materials, only with the further addition of cholesterol. The L-phase organisms thus differ in nutrient requirements from the pleuropneumonia group. They also differ in their requirements from the parent bacteria. Differences were noted in colonial appearances between the L-phase organisms studied and the organisms of the pleuropneumonia group.

It has been shown that many bacteria can exist in an L-phase which differs considerably in morphology and properties from the original bacilli (Klieneberger-Nobel, 1951; Dienes & Weinberger, 1951). Morphological studies have suggested close similarities between bacteria in the L-phase and organisms of the pleuropneumonia group, and it has been stated that cultures of the two may be indistinguishable (Tulasne, 1951; Dienes & Weinberger, 1951). However, in an investigation of the biological properties of the pleuropneumonia group significant differences in cultural appearances were noted between the L-phase of *Streptobacillus moniliformis* and organisms of the pleuropneumonia group (Edward, 1950). The relationship of the pleuropneumonia group of organisms to the L-phase of bacteria would seem to require further investigation in view of its importance in determining the taxonomic position of the pleuropneumonia-like organisms.

Although cultures of the L-phase of certain Gram-positive bacteria have been obtained on media not enriched with serum, the L-phases of Gram-negative bacteria have only been grown on media enriched with serum or ascitic fluid. All except the saprophytic members of the pleuropneumonia group require media similarly enriched. Cholesterol (or another sterol) has been shown to be an essential growth factor for the pleuropneumonia group; good growth was obtained on media in which serum was replaced by cholesterol + serum albumin or cholesterol + a phospholipid material from egg-yolk (Edward & Fitzgerald, 1951). The examination of the L-phases of two Gram-negative bacteria, to find whether serum could be replaced in the medium by the same substances, has now revealed differences in growth requirements between them and organisms of the pleuropneumonia group.

MATERIALS AND METHODS

Organisms. Three L-phase cultures were examined, namely, the L-phase of *Proteus vulgaris* (obtained from Dr L. Dienes), the L1 organism (isolated in this laboratory from *Streptobacillus moniliformis*) and the L-phase of a strain of salmonella (isolated in this laboratory from a culture of salmonella provided by Dr L. Dienes). The L-phase of salmonella only grew with difficulty and it was impossible to examine its growth requirements. Comparative observations were also made on four members of the pleuropneumonia group: *Asterococcus mycoides** and strains H17, C27 and C48 (Edward & Fitzgerald, 1951).

Media and supplements. The media and the methods for preparing suspensions of cholesterol and of the acetone-insoluble lipid fraction of egg yolk (AIL) have been described previously (Edward, 1947; Edward & Fitzgerald, 1951). The basal medium consisted of an ox heart infusion agar, to which was added 1% (w/v) peptone and 10% (v/v) yeast extract (50 mg. Oxoid yeast extract/ml.). The AIL was further purified by two successive precipitations with acetone from solution in chloroform.

Inoculation. Suspensions of L-phase organisms for seeding on experimental media were obtained by washing the growth from a serum agar plate into broth. The organisms were sedimented by centrifugation in an angle centrifuge (c. 3500 r.p.m.) and resuspended in broth. Serial ten-fold dilutions of suspension were inoculated on segments of a plate. Before inoculation with the L-phase of *Proteus vulgaris* the medium was treated by spreading over its surface four drops of a solution of penicillin containing 10,000 units/ml. to prevent reversion of the organism to the bacillary phase.

RESULTS

Nutritional requirements of L-phase and parent organisms

The results of the growth experiments are summarized in Table 1.

Strep. moniliformis. There was a trace of growth of the bacillus on the basal medium alone, which was not improved by adding 5 mg. bovine albumin (fraction V, Armour Laboratories)/ml. of medium, or cholesterol (0.1 mg./ml.). Growth equal to that on a medium enriched with 10% (v/v) horse serum was obtained by adding albumin+cholesterol. There was a moderately good growth on a medium containing AIL alone and growth about equal to that on serum agar on a medium containing AIL+albumin. Heilman (1941) showed that for growing *Strep. moniliformis* serum could be replaced in a fluid medium by starch. Dumhoff & Duffy (1951) found dextrin and glycogen also capable of replacing serum; they suggested that these carbohydrates might act by neutralizing an inhibitory substance. Growth in the media containing carbohydrates was not as good as with serum.

L-phase of *Strep. moniliformis* (L1). The L-phase did not grow on the

* The organism causing contagious bovine pleuropneumonia; in previous papers it was erroneously called *Asterococcus bovis*.

basal medium alone. It grew moderately well after the addition of bovine albumin (5 mg./ml.); colonies were as numerous as on serum agar but were small and variable in size. There was also a moderate growth when AIL (0.8 mg./ml.) was added to the basal medium. Growth equal to that on serum agar was obtained by adding bovine albumin + AIL. With a larger amount of

Table 1. *Summary of results of growth on experimental media*

Additions to basal medium	Organisms of the pleuropneumonia group				L-phase organisms		<i>Streptobacillus moniliformis</i>
	Strain C27	Strain C48	Strain H17	<i>Astero-coccus mycoides</i>	<i>Proteus</i> 'L'	L1	
No addition	—	—	—	—	—	—	tr
Bovine albumin (5 mg./ml.)	—	—	—	—	±	±	tr
AIL (0.8 mg./ml.)	—	—	—	—	+	±	±
Albumin and AIL	—	—	—	—	+	+	+
Cholesterol (0.1 mg./ml.)	tr	tr	—	—	—	—	tr
Cholesterol and albumin	+	±	+	tr	tr	±	+
Cholesterol and AIL	+	±	+	±	tr	±	±
Cholesterol, albumin and AIL	+	+	+	+	±	±	±

* Using 2 mg./ml. of AIL; not such good growth with 0.8 mg./ml.

+ = good growth; ± = poor growth; tr = trace of growth only; — = no growth.

AIL (2 mg./ml.) growth was not as good. There was no growth in a medium to which cholesterol (0.1 mg./ml.) had been added. Cholesterol appeared to be inhibitory, because the addition of this amount of cholesterol to media containing bovine albumin alone, AIL alone or albumin + AIL usually gave poorer growth than in the corresponding medium without cholesterol.

Pr. vulgaris. This organism is known to be capable of growing on a chemically-defined medium containing only ammonia lactate, nicotinic acid and salts (Fildes, 1938).

L-phase of *Pr. vulgaris* (*Proteus* 'L'). There was no growth on the basal medium alone. After the addition of bovine albumin (5 mg./ml.) there was a moderate growth, the colonies varying in size and being smaller and less numerous than on serum agar. Growth about equal to that on serum agar was obtained by adding AIL (0.8 mg./ml.). The organism failed to grow on a medium containing cholesterol (0.1 mg./ml.), and the addition of cholesterol to media containing albumin alone, AIL alone, or albumin + AIL, gave growth which was poorer than on the corresponding medium without cholesterol.

Nutrient requirements of the pleuropneumonia group of organisms

The growth of organisms of the pleuropneumonia group on media containing cholesterol, bovine albumin and AIL, singly and in combination, has been tested previously (Edward & Fitzgerald, 1951). Cholesterol was found to be a necessary medium component, and it appeared that both lipid and protein fractions of serum were concerned in its ability to promote growth. However,

in a report published later Smith & Morton (1951) reached a different conclusion regarding the nature of the growth factor in serum and ascitic fluid; they believed it to be a protein of low molecular weight or a large polypeptide. They obtained good growth of human genital strains by replacing the serum of their medium by a fraction of serum protein, without adding cholesterol or any lipid. In view of this divergence in results the experiments have been repeated in this laboratory. Confirmation has been obtained of failure to obtain growth on the basal medium used, unless cholesterol was added. Four strains of organisms of the pleuropneumonia group were tested. Two of the four grew poorly on a medium to which cholesterol (0.1 mg./ml.) only had been added (Table 1). On a medium containing cholesterol + bovine albumin (5 mg./ml.) three strains grew well or moderately well, there being only a trace of growth with *Asterococcus mycoides*. All four strains gave a moderate or good growth on a medium containing cholesterol + AIL, and grew as well as on serum agar when cholesterol + albumin + AIL were incorporated in the medium. There was no growth with albumin alone, AIL alone or albumin + AIL without cholesterol.

Attempts were made to reconcile these findings with those of Smith & Morton (1951). Fractions A and B, prepared from horse serum according to the methods they described, were incapable of adequately supporting the growth of pleuropneumonia-like organisms. Good growth was obtained using a commercially-available serum fraction (Bacto-PPLO serum fraction, Difco Laboratories), stated to be the same as Smith & Morton's fraction A. From 100 ml. of this fraction 650 mg of ether-soluble material was extracted by Hartley's method (1925). This material gave a strong Liebermann-Burchard reaction, and the original serum fraction was estimated to contain at least 150 mg. cholesterol/100 ml. The serum fraction after extraction (protein fraction) no longer supported growth (Table 2). The ether-soluble material (lipid fraction) alone also failed to support growth, but good growth was obtained by adding both protein and lipid fractions to the medium.

There was moderately good growth on a medium containing cholesterol + the

Table 2. *Growth with Bacto-PPLO serum fraction after separation into its lipid and protein fractions*

Additions to basal medium	Strains of organisms of the pleuropneumonia group			
	C27	C48	H17	<i>Asterococcus mycoides</i>
Bacto-PPLO serum fraction (1 %)	+	+	+	±
This fraction after removal of lipids (protein fraction) (5 %)	—	—	—	—
Extracted lipid fraction (5 %)	—	—	—	—
Protein fraction (5 %) + lipid fraction (5 %)	+	+	+	±
Protein fraction (2 %) + cholesterol (0.1 mg./ml.)	+	±	±	±
Lipid fraction (5 %) + bovine albumin (5 mg./ml.)	+	+	+	tr

Protein and lipid fractions were reconstituted to the original volume of the material fractionated.

protein fraction and on a medium containing the lipid fraction + bovine albumin. These results are in agreement with those obtained previously.

DISCUSSION

When an organism passes from the bacillary to the L-phase its growth requirements are radically changed. It becomes more exacting in its requirements so that subculture is always difficult; often attempts at subculture fail completely. Some aerobic bacteria, such as *Salmonella* spp., require low oxygen tensions in the L-phase. Media enriched with serum or ascitic fluid are needed to grow the L-phase of Gram-negative bacteria. In the present work a mixture of bovine albumin + a lipid fraction of egg yolk satisfactorily replaced serum for growing proteus 'L' and L1. *Pr. vulgaris* in the bacillary phase can grow on a chemically-defined medium containing ammonium lactate and nicotinic acid. The requirements of *Streptobacillus moniliformis*, the parent organism of L1, are more complex. It needed media enriched with serum, there being only a trace of growth on the basal medium alone. Serum could be replaced by bovine albumin + cholesterol; in this respect the organism differed from its L-phase. The more or less complete resistance of all L-phase organisms to the bacteriostatic action of penicillin would appear to reflect a fundamental difference in metabolism from that of the parent bacilli.

Oily globules are conspicuous in colonies of L1 examined microscopically. Partridge & Klieneberger (1941) showed that they consisted largely of cholesterol. They were able to extract from the organisms an amount of cholesterol which represented about 16% of their dry weight. It appeared that the cholesterol was not synthesized by the organism but came from the medium. No growth occurred on a medium in which serum was replaced by cholesterol or cholesteryl palmitate. The L1 organism can grow in a medium enriched with bovine albumin instead of serum. Cholesterol could not be detected in bovine albumin by chemical estimation. Colonies of the second of two serial subcultures of L1 on this medium were examined microscopically after 6 days' incubation; no oily globules were seen. It thus appears that cholesterol is not needed for the growth of L1, but when present in the medium, tends to be accumulated or released from some solubilized form by the organism.

Morphological studies have tended to emphasize a close relationship between bacteria in the L-phase and organisms of the pleuropneumonia group (Dienes & Weinberger, 1951). All except the saprophytic species of the latter group require media enriched with serum or ascitic fluid. The serum can be replaced by cholesterol + bovine albumin + a phospholipid fraction (AIL) of egg yolk. It has not been possible in this laboratory to obtain growth except on media containing cholesterol or certain other sterols. On the other hand the two L-phase organisms studied grow well without the addition of cholesterol. These findings need to be confirmed by a study of the L-phase of other bacteria, but suggest that there may be important metabolic differences between the L-phase of bacteria and organisms of the pleuropneumonia group, the latter organisms requiring cholesterol while the L-phase organisms either do not require it or are able to synthesize it.

Similarities between bacteria in the L-phase and organisms of the pleuropneumonia group have possibly been over-emphasized. Fully-grown cultures of most L-phase organisms, according to published descriptions, differ considerably in their colonial appearances from organisms of the pleuropneumonia group. Colonies of adapted strains of the different species of the pleuropneumonia group are remarkably alike; they are circular in outline, circumscribed and translucent, with delicate surface markings and are usually small. In comparison, colonies of L1 were more opaque and had coarser markings (Edward, 1950). In semi-solid and fluid medium the growth of L1 was more like that of bacteria. The colonies of the L-phase of *Proteus vulgaris* and *Salmonella* sp. were also relatively opaque and had coarse surface markings. It would thus appear that a bacterium in the L-phase tends to combine in the appearances of its growth the characters it possessed as a bacillus with the characters of a pleuropneumonia-like organism.

Smith & Morton (1951) claimed that a protein of low molecular weight, capable of separation from whole serum, alone was responsible for the ability of serum to promote growth of organisms of the pleuropneumonia group. Their findings are thus in apparent conflict with the work which suggests that cholesterol is an essential growth factor. The experiments with cholesterol were repeated and confirmed previous findings. A commercially-available preparation of Smith & Morton's fraction was found in fact to contain considerable amounts of lipid, including cholesterol. It may therefore be questioned whether the activity of the fractions examined by Smith & Morton and believed by them to consist 'of proteinaceous material only', was not due to their containing lipid. In reporting a subsequent extension of their work Smith & Morton (1952) made no reference to the evidence regarding cholesterol. It would have been of value to have had their explanation for the divergent experimental findings and to have learnt whether they had examined their active preparations for the presence of cholesterol, as we have done.

I wish to thank Dr L. Dienes for supplying me with cultures, Prof. B. C. J. G. Knight for his encouragement and advice and Dr A. J. Woiwod for cholesterol estimations.

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(Received 18 August 1952)

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Isolation of Anaerobic Bacteria by a Modified Shake Method

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SUMMARY: Isolated colonies of anaerobic bacteria can be obtained in a layer of agar enclosed between a watch-glass and a glass plate. They can be examined and removed without difficulty.

The excuse for introducing yet another way of isolating anaerobic bacteria is that it possesses some advantages over existing methods and should prove of value in small laboratories not provided with special apparatus. In deep nutrient agar, discrete, well-developed colonies can be obtained with regularity but their examination and removal can be difficult. This can be overcome by cultivating the microbes in a layer of agar contained between two plates of glass clamped on a U-shaped metal support (Mason, 1930). Unfortunately, because the edges of the agar are then exposed to air, contamination and shrinkage of the medium is the rule with this method. These disadvantages are removed in the method to be described.

The apparatus required consists of watch-glasses of between 5 and 10 ml. capacity, glass plates about 10 cm. square, nutrient agar, serum, drawn-out pipettes, a platinum loop, and paraffin wax. The borders of the watch-glasses on the concave side are ground flat so that they will adhere firmly to the plates. The glasses and plates are wrapped in paper and sterilized by moist or dry heat. Nutrient agar in tubes is melted, cooled to 50° and serum added to give a 10% (v/v) concentration. The microbes under test are added and thoroughly dispersed. The inoculated medium is then poured into the watch-glass held under, and projecting slightly beyond the edge of, the horizontal glass plate. As the watch-glass fills, it is slowly slipped right on to the plate, but an air bubble is allowed to take the place of the last drop of agar so as to avoid overflow. The plate is placed on edge to allow the agar to set with the bubble at the circumference of the watch-glass. After ringing the watch-glass with wax, the apparatus is incubated at 37°.

The serum agar must be clear, firm and capable of supporting the growth of the anaerobe under examination. Fermentable sugar should not be added, in order to minimize gas formation; in fact, it may be necessary to remove some of the carbohydrates from the mother broth by fermentation methods if gas formation is troublesome. Colonies, visible to the eye, develop within 24 hr. The wax is then removed and the watch-glass levered off with a sterile thin-bladed knife. Then, under a low power microscope or under a lens, the chosen colony is sucked into a pipette and expelled into broth.

Excellent results have been obtained with *Clostridium tetani*, *Cl. chauvoei*, *Cl. septicum* and *Cl. oedematiens*. *Cl. chauvoei* has been isolated from a mixture

of *Cl. chauvoei* and *Cl. septicum* and from a mixture of *Cl. chauvoei* and *Cl. oedematiens*. It will be appreciated by those accustomed to the isolation of anaerobes that this method is but a refinement of an existing technique and is not a solution of all the difficulties inherent in this type of work. To expect to isolate *Cl. chauvoei* from a culture grossly contaminated with *Cl. sporogenes*, *Bacterium coli* and a staphylococcus is asking too much. All means at the disposal of the technician, such as differential heating, cultivation in selective media and passage through small animals, should be used to bring to a minimum the number of concomitant bacteria before colony isolation methods are applied.

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(Received 18 August 1952)

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The Effect of Aeration on the Utilization of Respiratory Substrates by *Penicillium chrysogenum* in Submerged Culture

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SUMMARY: At high aeration rates in submerged culture, lard oil (mainly glycerides of oleic and stearic acids) may be utilized by *Penicillium chrysogenum* as a source of carbon in preference to carbohydrate. At relatively low aeration rates, carbohydrate serves as the principal carbon source.

Enzymes involved in the utilization of lard oil appear to be synthesized by the growing mycelium, this synthesis being favoured by conditions of high aeration.

In an earlier paper (Rolinson, 1952) it was shown that the degree of aeration in spore-inoculated penicillin fermentations affects not only the rate of respiration but also the kind of metabolism carried out. In shaken flask fermentations, the R.Q. of the mycelium was *c.* 1.0 throughout the fermentation, whereas in fermentations at very high aeration rates the R.Q. of the mycelium was *c.* 1.0 only during the first 30 hr., then fell to values as low as 0.6 as the fermentation proceeded. The R.Q. values for the two types of mycelium were little affected by the immediate degree of aeration. Thus mycelium grown at low aeration rates continued to respire with an R.Q. of *c.* 1.0, even though an excess of dissolved oxygen was present, and conversely the low R.Q. of the mycelium from the highly aerated fermentations was still evident when respiration was limited by the concentration of dissolved oxygen. The differences in metabolism, as shown by the different R.Q. values, therefore appeared to indicate the presence of different enzyme systems in the mycelia, having presumably been developed as a result of the differences in concentration of dissolved oxygen during growth.

The metabolism of mycelium of *Penicillium chrysogenum* has been studied further, and the present paper concerns the respiratory substrates utilized by the two types of mycelium and which are responsible for the differences in the R.Q. observed previously.

METHODS

Fermentation methods

Laboratory fermenters of the type described by Lumb & Fawcett (1951) using 3 l. of medium were operated at different rates of stirring and air flow. Shaken flask fermentations were carried out with 75 ml. lots of medium in 250 ml. conical Pyrex or Hysil flasks on a rotary shaker (speed 220 r.p.m.; throw of 1.5 in.). All fermentations were carried out at 24-26°.

The medium used in all the experiments contained (% w/v): corn steep liquor (solids basis), 1.5; lactose, 3.0; glucose, 0.5; NaNO₃, 0.3; KH₂PO₄,

0.05; Na_2SO_4 , 0.1; pH value adjusted to 5.8. Phenylacetic acid was added to the stirred and aerated fermentations in additions of 0.05 % (w/v) every 12 hr., starting at the 24th hr. In the shaken flask fermentations, a concentration of 0.25 % (w/v) phenylacetic acid was added to the medium initially. Lard oil containing 2.5 % (v/v) cetyl alcohol was used as an antifoam agent as required. The lard oil, which consists mainly of glycerides of oleic and stearic acids, was obtained from Messrs Brown and Deighton, Preston, Lancashire. All the fermentations were inoculated with spore suspensions prepared from lyophilized master cultures of *P. chrysogenum* W48-701.

Analytical methods

Glucose and lactose were estimated by determining the reducing power of the sample before and after hydrolysis. The difference between the two values was taken to be equivalent to half the quantity of lactose present in the sample from which the separate quantities of glucose and lactose were calculated. The sample was hydrolysed by boiling with *N*-HCl for 45 min. Reducing power was estimated by the following modification of the Folin-Wu (1920) method. The sample was filtered through kieselguhr and diluted so that 3 ml. contained less than 0.8 mg. glucose. A 3 ml. sample of this solution was boiled for 6 min. with 2 ml. of Folin's alkaline cupric solution in a Folin tube. The tube was then placed in cold water for 1 min. After boiling again for 2 min. with 2 ml. of Folin's phosphomolybdate solution the tube was again placed in cold water for 1 min. before being made up to the 25 ml. mark with distilled water. The contents of the tube were then mixed and read immediately in a Spekker absorptiometer using yellow filters (Hilger 606). Reducing power was read from a calibration curve prepared with standard solutions of glucose. During boiling with the phosphomolybdate solution, small glass funnels were placed in the necks of the Folin tubes to prevent reoxidation by the atmosphere.

Total Kjeldahl nitrogen was determined by the conventional procedure using a Markham distillation apparatus; *amino-nitrogen* was determined by Sorensen's formol titration method; *mycelial dry weight* was determined as described by Rolinson (1952). Conventional Warburg manometric techniques were used as described by Umbreit, Burris & Stauffer (1945).

RESULTS

Utilization of carbohydrates

The usage of glucose and lactose in fermentations at high aeration rates and in fermentations in shaken flasks (relatively low aeration) is shown in Figs. 1 and 2. It will be seen that in the shaken flask fermentations glucose and lactose were both used rapidly and completely, the residual carbohydrate at the end of the fermentation being usually negligible. In the highly aerated fermentations however, although the glucose was again completely used, lactose was only utilized rapidly during about the first 30 hr. After this time the rate of lactose utilization was slow, and at the end of fermentation usually as much as 60 % of the initial lactose remained in the fermentation medium.

It has been shown (Rolinson, 1952) that extremely high rates of solution of oxygen are obtained in these laboratory fermenters as compared with the rates in shaken flasks, and it therefore appeared from the results shown in Figs. 1 and 2 that aeration above the degree obtained in shaken flasks inversely affected the rate of lactose usage. Accordingly, fermentations were carried out in the

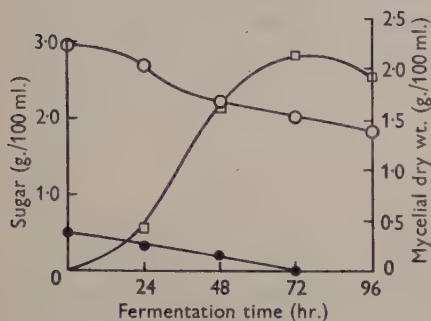


Fig. 1

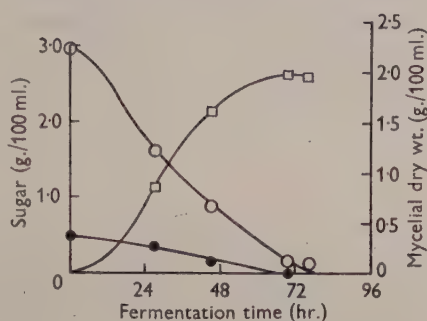


Fig. 2

Fig. 1. Growth and usage of lactose and glucose in a highly aerated fermentation. Stirring 1000 r.p.m., air flow 2 vol./vol./min. Mycelial dry wt., \square — \square ; lactose, \circ — \circ ; glucose, \bullet — \bullet .

Fig. 2. Growth and usage of lactose and glucose in a shaken flask fermentation. Mycelial dry wt., \square — \square ; lactose, \circ — \circ ; glucose, \bullet — \bullet .

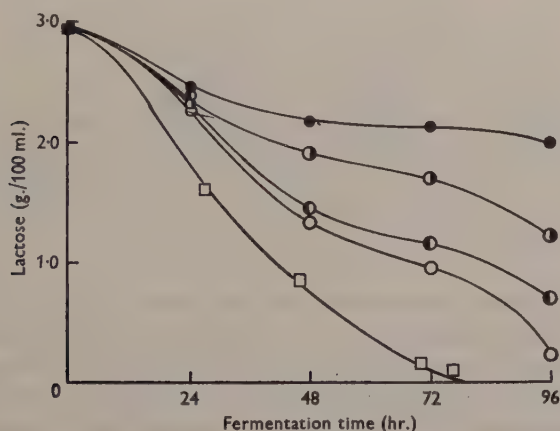
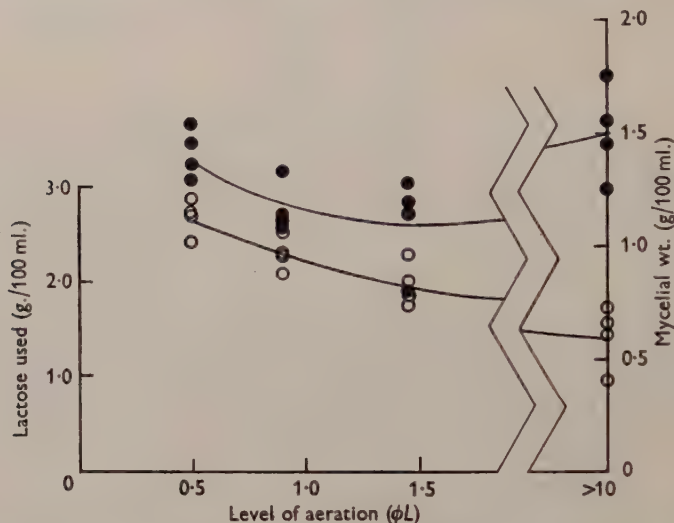


Fig. 3. Effect of rates of stirring and air flow on the usage of lactose in fermentations in laboratory fermenters. 1000 r.p.m. and 2 vol./vol./min., \bullet — \bullet ; 1000 r.p.m. and 0.3 vol./vol./min., \bullet — \bullet ; 500 r.p.m. and 0.5 vol./vol./min., \bullet — \bullet ; 500 r.p.m. and 0.3 vol./vol./min., \circ — \circ . Shaken flask fermentation, \square — \square .

laboratory fermenters under different conditions of aeration, obtained by varying the rates of stirring and air flow (see Fig. 3), which resulted in progressively altered rates of solution of oxygen in the fermentation medium as determined by the method of Wise (1951). The usage of lactose under different degrees of aeration thus obtained is shown in Fig. 3. It will be seen that increased aeration resulted in diminished rates of lactose usage, the lowest

rate occurring with the highest degree of aeration. Lactose usage was also depressed when the degree of aeration was below a certain value. By increasing the volumes of medium used in the shaken flasks the rate of solution of oxygen could be so decreased that both lactose usage and the amount of mycelium formed were markedly diminished. The incomplete usage of lactose under conditions of high aeration, however, was not accompanied by a corresponding diminution in the amount of mycelial growth. This suggested that, under con-



quantity to provide the necessary carbon for the amount of growth made when lactose usage was very incomplete were the proteins and amino-acids of the corn-steep liquor and the lard oil added as a vehicle for the antifoam agent. The quantity of lard oil thus added frequently amounted to as much as 1% (v/v).

Utilization of nitrogenous constituents of the medium

The usage of the organic nitrogenous constituents of the medium was examined, and it was found that their utilization was not significantly influenced by the degree of aeration. The latter substances therefore did not appear to be the alternative carbon source utilized under conditions of high aeration.

Utilization of lard oil

The total amount of lard oil added during two separate fermentations (c. 1.0%, v/v) was recorded, and the residual fat extracted from the broth at the end of the fermentation by shaking 500 ml. volumes with two successive 100 ml. volumes of chloroform. The chloroform extracts were combined with chloroform washings of the interior of the fermenter, evaporated and weighed. The results are shown in Table 1. It will be seen from these results that only c. 5% of the total lard oil added was recovered at the end of the fermentation.

Table 1. *Recovery of lard oil added during highly aerated penicillin fermentations*

	Stirring 1000 r.p.m., air flow 2 vol./vol./min.		
	Lard oil added during the fermentation (g.)	Lard oil extracted after fermentation (g.)	Lard oil recovered (%)
Exp. 1	25.8	1.25	4.75
Exp. 2	25.7	1.36	5.31

Evidence that lard oil could be rapidly oxidized was obtained from manometric experiments with washed mycelium.

Respiration of mycelium from highly aerated fermentations. Samples of fermentation brew were removed and the mycelium washed four times by centrifuging and suspending in a solution containing only the inorganic constituents of the medium (salts solution). The concentration of mycelium in these suspensions was similar to that in the fermentation at the time of sampling. The pH value of the salts solution was also adjusted to that of the fermentation at the time of sampling. Oxygen uptake was measured in Warburg manometers at 26° rocked at 120 strokes/min. Each flask contained 1.0 ml. of mycelial suspension together with a further 1.0 ml. of the salts solution to prevent the mycelium from becoming too densely packed in the flask.

The respiration of washed 46 hr. mycelium from a high aeration rate fermentation, in the presence of various substrates, is shown in Fig. 5. The relatively high endogenous rate of respiration, which is typical of fungi, was not diminished by further washing. It will be seen from Fig. 5 that not only

was lard oil oxidized rapidly but the rate of respiration with lard oil as substrate was higher than with glucose or complete fermentation medium.

Respiration of mycelium from shaken flask fermentations. Mycelium 69 hr. old from shaken flask fermentations (relatively low degree of aeration) was washed and suspended in salts solution as before and respiration measured in the presence of different substrates. It will be seen from Fig. 6 that although glucose was oxidized rapidly, the presence of lard oil did not result in any

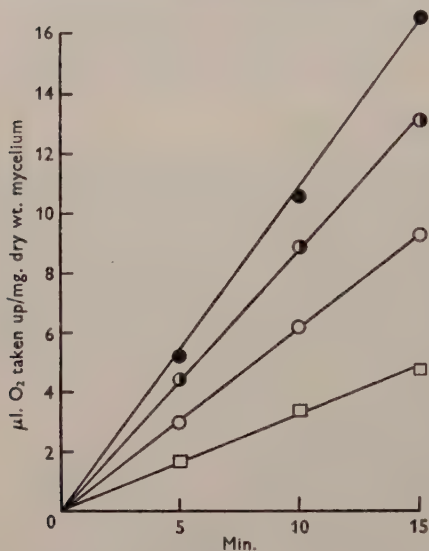


Fig. 5

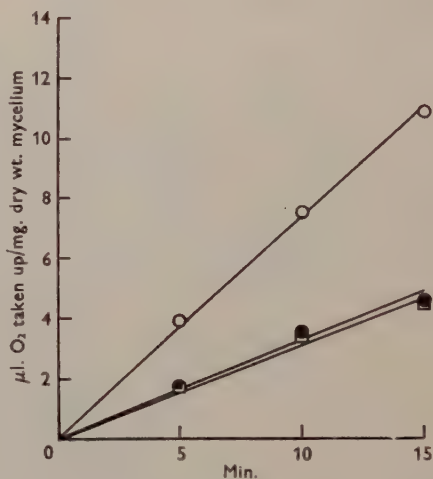


Fig. 6

Fig. 5. Respiration of washed, 46 hr. mycelium of *P. chrysogenum* from a fermentation carried out in a laboratory fermenter stirred at 1000 r.p.m. with an air flow of 2 vol./vol./min. Substrates: 1% (v/v) lard oil, ●—●; complete sterile fermentation medium, ●—●; 1% (w/v) glucose, ○—○. Endogenous respiration, □—□.

Fig. 6. Respiration of washed 69 hr. mycelium of *P. chrysogenum* from a shaken flask fermentation. Substrates: 1% (v/v) lard oil, ●—●; 1% (w/v) glucose, ○—○. Endogenous respiration, □—□.

significant increase in respiration above the endogenous rate with mycelium from low aeration flasks. Thus if lard oil was metabolized by mycelium from shaken flasks it was certainly oxidized at a very much lower rate than by mycelium from the fermentations at high aeration rate. Since all the manometric experiments were carried out in flasks rocked at the same rate of 120 strokes/min., the difference in oxidation of lard oil by the two types of mycelium appeared to indicate a difference in enzyme content.

DISCUSSION

The effect of high aeration rates on lactose usage appears to be peculiar to fermentations inoculated with spores. In fermentations starting with a vegetative inoculum and with lard oil as vehicle for the antifoam agent, lactose was used rapidly and completely even at the highest degrees of aeration. There is,

indeed, evidence that the usage of lactose is favoured by high aeration rates in fermentations started by inoculation with vegetative growth (Bartholomew, Karow, Sfat & Wilhelm, 1950; Brown & Peterson, 1950*a*, *b*). In fermentations inoculated with vegetative growth, lard oil and related substances may also be metabolized as in spore-inoculated fermentations but evidently not in preference to carbohydrate.

The R.Q. values reported by Rolinson (1952) indicated a difference in metabolism between mycelium grown at high aeration rates and that grown at relatively low aeration rates. It is clear from the present results that one such difference lies in the relative ability of the two kinds of mycelium to oxidize lard oil. Only with the highest degree of aeration was lard oil utilized extensively and in preference to lactose. It is not clear whether high concentrations of dissolved oxygen directly inhibit the usage of lactose or whether the metabolism of lard oil involves some mechanism which is also involved in the utilization of lactose so that lard oil might diminish lactose usage by competition for this common mechanism. The results shown in Fig. 4 appear to indicate that increasing concentrations of dissolved oxygen do directly inhibit the utilization of lactose, since a limited increase in degree of aeration resulted in a fall in mycelial weight which corresponded to the fall in the amount of lactose used. Only when the aeration was increased to 1000 r.p.m. with air flow of 2 vol./vol./min. was some other substrate utilized efficiently as an alternative to lactose. Alternatively, the diminished uptake of lactose might nevertheless be due to competition with lard oil for some common mechanism, with the metabolism of lard oil serving in the synthesis of mycelium relatively inefficiently except under the highest degree of aeration.

Evidence has been presented (Rolinson, 1952) that differences in R.Q. values between mycelia from fermentations at different degrees of aeration represent a difference in enzyme content rather than a difference in response to the immediate condition of aeration. This is also borne out by the experiments described here. Mycelium from shaken flask fermentations showed no detectable oxidation of lard oil despite the excess of dissolved oxygen in the Warburg flasks, while these conditions permitted mycelium grown under conditions of high aeration to oxidize lard oil rapidly. These results suggest that enzymes involved in the metabolism of lard oil are synthesized during spore-inoculated penicillin fermentations and that the synthesis is favoured by conditions of high aeration.

The authors are indebted to the late Sir Jack Drummond, F.R.S., and Mr C. E. Coulthard for their interest in the work, to Mr R. Fawcett for assistance in carrying out the fermentations and Mr C. K. Mercer and Mr J. Ransom for technical assistance.

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(Received 19 August 1952)

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The Breakdown of Naphthalene by a Soil Bacterium

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SUMMARY: A Gram-negative motile bacterium isolated from soil can grow with naphthalene as sole carbon source and produces from it *D-trans*-1:2-dihydro-1:2-dihydroxynaphthalene and salicylic acid.

The decomposition of naphthalene in soil was studied by Tattersfield (1928) in connexion with its use as a soil insecticide. The disappearance of naphthalene from soil was shown to be due to bacterial action and was prevented by sterilizing the soil. Several strains of bacteria which could use naphthalene as a source of carbon were isolated from naphthalene-treated soils by Gray & Thornton (1928). At about the same time Tausson (1927) found three kinds of bacteria able to metabolize naphthalene in soils from the Black Sea oilfields. These bacteria would not grow on 1- or 2-naphthol, phthalic acid, catechol, hydroquinone, resorcinol, pyrogallol or phloroglucinol. It was concluded that none of these compounds was an intermediate in the breakdown of naphthalene and that probably both rings were split simultaneously. On the other hand, other bacteria which could grow on phenanthrene (but not on naphthalene) could use catechol, salicylic acid and saligenin but not resorcinol, phloroglucinol, pyrogallol or phthalic acid (Tausson, 1928). The oxidation of phenanthrene was supposed to proceed via 2-hydroxybenzyl alcohol, 2-hydroxybenzaldehyde, salicylic acid and catechol. Jacobs (1931) reported a positive fluorescein test for phthalic acid in a pure culture of a naphthalene-decomposing bacterium. In a note, Strawinski & Stone (1943) claimed to have found 'ortho-salicylic acid' in the acid ether extract from a culture of *Pseudomonas aeruginosa* grown on naphthalene. They gave no experimental details and we have been unable to trace any subsequent publication.

The simultaneous adaptation technique of Stanier (1947) was used in the present work as a means of deciding whether various compounds were intermediates in the bacterial metabolism of naphthalene.

METHODS

The naphthalene-decomposing organism was isolated from Rothamsted allotment soil by enrichment culture in a modified Lees & Quastel (1944) percolator, followed by plating on mineral agar with naphthalene as the only other carbon source. The percolator was charged with 20 g. of 3 mm. soil crumbs mixed with 0.25 g. sublimed naphthalene and perfused with tap water for 1 week.

Media. Tausson's (1927) medium was used with or without the addition of agar. It consists of two solutions which were sterilized separately and mixed aseptically in the proportion of 4 of (a) to 1 of (b). Solution (a) is: $\text{Ca}(\text{NO}_3)_2$, 1 g.; KNO_3 , 0.25 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g.; $\text{Fe}_2(\text{SO}_4)_3$, 0.005 g.; distilled water,

800 ml. Solution (b) is: KH_2PO_4 , 0.5 g.; K_2HPO_4 , 0.5 g.; distilled water, 200 ml.

Culture conditions. Plates of Tausson's medium solidified with Difco standardized agar (2%, w/v) were inoculated and inverted over crystals of freshly sublimed naphthalene contained in the lid. Cultures in liquid media were grown in 1 l. Roux bottles containing 125 ml. or in 2 l. conical flasks containing 500 ml. About 0.1 g. sterile naphthalene was added aseptically to each bottle after autoclaving. All cultures were incubated at 25°.

Naphthalene diols. DL-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene and the L-isomer were isolated from the urine of rabbits and rats, respectively, which had been given naphthalene. A pure specimen of L-diol was a gift from Prof. L. Young. The diols were estimated as 1-naphthol by the method of Boyland & Wiltshire (1953).

Manometric method. Oxygen uptake was measured in Warburg manometers. The organisms for these experiments were grown on agar plates for 4–5 days, washed twice with 0.02M-phosphate (pH 7.0) and resuspended in the same buffer.

RESULTS

Description of organism

The strain used was an aerobic motile Gram-negative rod which did not liquefy nutrient gelatin. On mineral salts agar+naphthalene the smooth circular colonies (6–8 mm. diam.) became coloured yellow and later brown. These properties do not distinguish it from the *Bacillus naphthalinicum non-liquefaciens* of Tausson (1928).

Detection and isolation of salicylic acid

Liquid cultures after 4–6 days showed a marked violet colour on adding dilute ferric chloride solution. Paper chromatography of these cultures by the method of Bray, Thorpe & White (1950) revealed a spot with the same R_f value as salicylic acid, 23 mg. of this compound were isolated from about 2 l. of acidified culture by extraction into ether. The ether extract was washed with water and with 2% (w/v) sodium bicarbonate solution. The aqueous layer was acidified with dilute hydrochloric acid, extracted with fresh ether, the ethereal layer separated, washed with water, dried over sodium sulphate, filtered and evaporated to dryness. The residue was crystallized from water and sublimed over a boiling water-bath; colourless needles were obtained, m.p. 156°; mixed m.p. with authentic salicylic acid 156–157°. On heating with a drop of methanol and sulphuric acid, the odour of methyl salicylate was detected.

Detection and isolation of naphthalene diol

Liquid cultures grown for about 2 days contained a substance which yielded 1-naphthol after treatment with N-hydrochloric acid for 15 min. at 100°. Practically no free naphthol was found at this stage of growth. The culture was neutralized with sodium bicarbonate, heated to 70° and filtered. The diol in 2 l. culture filtrate was adsorbed by stirring with 4 g. animal charcoal, the charcoal separated and eluted 3 or 4 times with hot ethanol. The combined

eluates were concentrated to dryness, under reduced pressure, below 40°. The residue was extracted with boiling benzene and the benzene solution evaporated to dryness. The residue (12 mg.) recrystallized from hot benzene yielded 9.5 mg. colourless needles, m.p. 125°. (Found: C, 73.6; H, 5.9; calc. for $C_{10}H_{10}O_2$; C, 74.1; H, 6.2.) Further recrystallization raised the m.p. to 127° (Kofler block). The spectrum of this substance in solution in 95 % (v/v) ethanol showed an absorption maximum at 263–4 m μ ; authentic DL-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene had a maximum at 264 m μ ; the spectra of naphthalene

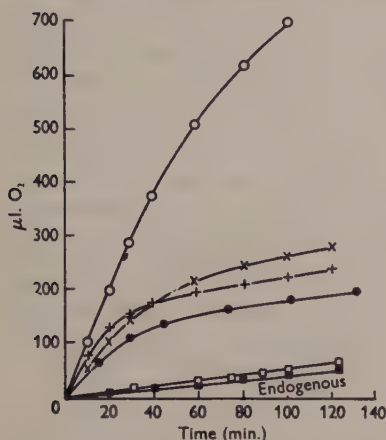


Fig. 1

Fig. 1. Rate of oxygen uptake of washed cells grown on naphthalene. In presence of: naphthalene (8.3 μ mole/3 ml.) (○—○), salicylate (2.5 μ mole/3 ml.) (×—×), DL-*diol*' (2.5 μ mole/3 ml.) (+—+), D-*diol*' (6.2 μ mole/3 ml.) (●—●), and L-*diol*' (5 μ mole/3 ml.) (□—□), as substrates.

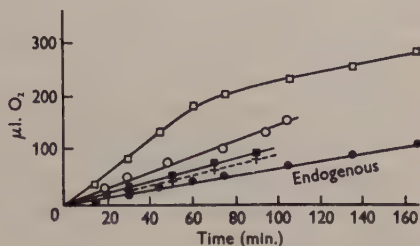


Fig. 2

Fig. 2. Rate of oxygen uptake of washed cells grown on naphthalene. In presence of: catechol (5 μ mole/3 ml.) (□—□), coumarin (2.5 μ mole/3 ml.) (■—■), 1:2-dihydroxynaphthalene (5 μ mole/3 ml.) (+—+), and β -naphthoquinone (1.3 μ mole/3 ml.) (○—○) as substrates.

and of 1- and 2-naphthol are different. The specific rotation ($\alpha_D^{25} + 161^\circ$ ($c = 0.8712\%$ in ethanol) compares with $+159^\circ$ ($c = 1\%$ in ethanol) found by Booth & Boyland (1949) for D-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene from urine of rabbits treated with naphthalene. Unfortunately no specimen of this compound was available for comparison, but mixtures of the bacterial product with L-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene from rat urine (m.p. 127°) melted at various temperatures between 99 and 120°.

Manometric experiments

The bacteria grown on naphthalene were found to be simultaneously adapted to oxidize salicylic acid, catechol, the D-*trans*-diol and the DL-*trans*-diol, but not the L-*trans*-diol, α -naphthoquinone, 3-hydroxybenzoic acid, *trans*-coumaric acid or 1-naphthol. There were small oxygen uptakes with coumarin, β -naphthoquinone and 1:2-dihydroxynaphthalene. These results are illustrated in Figs. 1 and 2.

DISCUSSION

In his work on the decomposition of phenanthrene Tausson (1927, 1928) decided that 2-hydroxybenzyl alcohol, salicylic acid and catechol were intermediates because they could serve as sole sources of carbon for growth and were not toxic; no such intermediates were established in the case of naphthalene. A pure culture of bacteria growing in a simple medium with naphthalene as sole carbon source presents a favourable system for a study of the breakdown of this hydrocarbon. We found that our organism did not grow on 1-naphthol, 2-naphthol or phthalic acid, but did grow on salicylic acid. When grown on naphthalene, washed cells showed an immediate oxygen uptake with the *D-trans*-diol, salicylic acid or catechol; the first two of these compounds were also isolated from growing cultures. The formation of catechol from salicylic acid by certain pseudomonads is known to occur (Walker & Evans, 1952).

The naphthalene diols have been known hitherto only as products of naphthalene metabolism in mammals where both *D*- and *L*-isomers are formed (Young, 1947; Booth & Boyland, 1949). Evidently they may also be produced at an early stage of oxidation of naphthalene by bacterial cells.

We are grateful to Prof. L. Young for the gift of *L-trans*-diol, to Prof. W. C. Evans for gifts of coumarin, *trans*-coumaric acid and α - and β -naphthoquinones, and to Dr D. E. Hathaway for determining the specific rotation of the diol.

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(Received 22 August 1952)

Chemotherapy and Plant Viruses

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SUMMARY: When the guanine analogue, 5-amino-7-hydroxy-1-v-triazolo (D) pyrimidine (guanazolo), was sprayed on the leaves of tobacco or *Nicotiana glutinosa* plants it reduced the number of local lesions and delayed or inhibited systemic spread of lucerne mosaic virus. Guanazolo was more effective when applied before inoculation, but had some effect if applied up to about the second day after inoculation. The compound was more effective in solution in 0.1% sodium bicarbonate than in aqueous suspension. With mechanically inoculated plants guanazolo watered on the soil around the plants was less effective than when sprayed on the leaves.

Incubated with the virus *in vitro* the compound did not affect infectivity. In concentrations up to c. 0.005M guanazolo usually caused negligible plant damage, but at higher concentrations produced a slight yellowing and distortion in the younger leaves with general stunting if treatments were prolonged.

The virus-inhibitory activity of guanazolo was reversed by adenine, guanine and possibly by hypoxanthine, but not by xanthine, uric acid, theobromine, theophylline, caffeine, uracil or thymine.

The triazolo analogue of adenine severely damaged plants and had only slight virus inhibitory activity. The hypoxanthine analogue caused no plant damage. It was less effective than guanazolo in tobacco and *N. glutinosa* but more effective in reducing the number of local lesions produced in beans. Thiouracil, methylthiouracil and propylthiouracil were ineffective against lucerne mosaic virus. Thiouracil caused fairly severe plant damage.

Guanazolo had slight or negligible effects on spotted wilt virus in tomato, potato viruses X and Y in potato, and tobacco and pea mosaic virus in peas. Applied as a leaf spray at 0.01M concentration guanazolo delayed or prevented systemic movement of cucumber mosaic virus from mechanically inoculated cucumber leaves, but had no effect when watered on the soil. However, watering the compound on the soil gave some control when the virus was introduced by aphids.

The indirect measures at present used to control plant-virus diseases, such as eliminating sources of infection and developing resistant varieties, are for many diseases only partially effective. The development of compounds which delay or inhibit virus multiplication within the plant could form a basis for a more widely applicable method of control. A variety of compounds, mainly antibiotics, plant-growth substances and dyestuffs, has been tested for possible antiviral activity. Takahashi (1948), using a detached leaf technique, found that malachite green decreased the amount of tobacco mosaic virus produced in *Nicotiana glutinosa* leaf tissue. Stoddard (1947) stated that the virus causing X disease of peach could be inactivated in living peach buds by soaking buds in solutions of a variety of compounds, and that trees watered or injected with various compounds could be protected against disease. Limasset, Levieil & Sechet (1948) found that 2-methyl-4-chlorophenoxy acetic acid temporarily inhibited the development of potato viruses X and Y in tobacco.

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Locke (1948) found that 2:4-dichlorophenoxyacetic acid masked the symptoms of leaf roll in a Netted Gem potato plant. He considered that the amount of active virus was also reduced. Kutsy & Rawlins (1950) found that naphthalene acetic acid decreased the amount of tobacco mosaic virus in tobacco tissue culture. Manil (1947) and Beale & Jones (1951) failed to obtain any control of tobacco mosaic virus or potato yellow-dwarf with a range of antibiotics. Ryjkoff & Smirnova (1948) showed that the multiplication of tobacco mosaic virus was depressed when inoculated leaves of *N. glutinosa* were immersed in 0.1 % magnesium sulphate solution or when tobacco leaves were infiltrated with 0.05 % tryptaflavine solution. Thomas & Baker (1949), working with a mosaic disease in carnations, stated that virus activity was reduced when plants were treated with several compounds, including a number of sulphonamides. Köhler & Hauschild (1950) found that sodium sulphide, potassium hydroxide, potassium permanganate, and ascorbic acid had no effect on potato leaf roll. Nickell, Greenfield & Burkholder (1950) described the effects of various nucleic acid components and related compounds on virus tumours in *Rumex*, and Nickell (1951) showed that methylene blue, crystal violet and malachite green inhibited the growth of these tumours. Commoner & Mercer (1951) found that thiouracil inhibited multiplication of tobacco mosaic virus in tobacco leaf tissue.

It is usually assumed and has been shown for one plant virus (Markham & Smith, K. M. 1949) that the nucleic acid of the virus nucleoprotein is necessary for infectivity. In considering the possibility that analogues of the nucleic acid bases might be effective against plant viruses, our attention was drawn to the work of Kidder, Dewey, Parks & Woodside (1949) on the effects of guanazolo and other substituted purines on the metabolism of *Tetrahymena* and on the development of malignant cells in mice. The present paper describes tests with a number of these substituted purines and three analogues of uracil against several plant viruses. Preliminary reports of some of these results have already appeared (Matthews, 1951, 1952).

MATERIALS AND METHODS

Plants were grown in 4 in. pots in the glasshouse in a steam-disinfected potting mixture containing approximately 57 % loam, 33 % rotted organic matter and 10 % coarse sand. Lucerne mosaic virus was obtained from white clover (Fry, 1953); cucumber mosaic virus from a field infected tomato plant. The plants used were *Nicotiana glutinosa*, *N. tabacum* var. White Burley, *Phaseolus vulgaris* var. Sydney Wonder, *Trifolium subterraneum* var. Tallarook and the Short Prickly variety of cucumber. In *Nicotiana glutinosa* and *N. tabacum* lucerne mosaic virus produces etched ringspot (Pl. 1, fig. 1) or solid necrotic local lesions after 3–6 days, followed by systemic vein-clearing and etched mottling with some necrosis. In *N. glutinosa* it distorts leaves and stunts growth, particularly in the early stages of systemic spread (Pl. 1, fig. 2). In beans the virus produces brown necrotic local lesions. In the variety of subterranean clover used the virus produces a severe disease leading to death of the plants.

N. glutinosa plants were inoculated when c. 2–3 in. high with 3–4 expanded leaves; tobacco when it had two or three fairly well-expanded leaves and beans when the primary pair of leaves was fully developed. In each experiment plants were selected for uniformity of size and appearance. In placing plants in groups for treatments most of the remaining variation in size was kept within treatments. In all mechanical inoculations fine-grade carborundum powder was sprinkled on the leaves before inoculation. Unless otherwise noted dilutions of infective sap were made in distilled water and leaves were not washed after inoculation.

Compounds

5-amino-7-hydroxy-1-v-triazolo (D) pyrimidine (Guanazolo),
7-hydroxy-1-v-triazolo (D) pyrimidine (hypoxanthine analogue),
7-amino-1-v-triazolo (D) pyrimidine (adenine analogue),
5-hydroxy-7-amino-1-v-triazolo (D) pyrimidine,
5:7-dihydroxy-1-v-triazolo (D) pyrimidine,
2-hydroxy-6-aminopurine,
5-amino-7-hydroxy-(8, 1, 2)-oxadiazolo-(5, 4*d*) pyrimidine,
2:6-diaminopurine
(all given by the American Cyanamid Company);
2-aza adenine (given by Merck and Co.),
thiouracil, 6-methylthiouracil and propylthiouracil (Fisher Scientific Company),
adenine, guanine, thymine, uracil, hypoxanthine, caffeine, xanthine and theophylline (Light and Co.),
and uric acid and theobromine (British Drug Houses Ltd.).

Purity of compounds. The purity of the compounds used in reversal experiments was tested by paper chromatography. Two solvents were used—that of Marshak & Vogel (1951) and that of Markham & Smith, J. D. (1949). Reflex prints with no. 50 document paper were made of chromatograms under ultraviolet light with a Hanovia lamp and a filter as described by Markham & Smith, J. D. (1949).

Adenine and guanine could be adequately separated by the tertiary butanol system. The first batch of commercial guanine used in reversal experiments was shown to contain about 23 % (determined by elution from chromatogram and estimation in a Beckman spectrophotometer) of a compound moving with the same R_f as adenine. A second batch used in later experiments showed no evidence of such impurity. Resolution of hypoxanthine and adenine was not obtained with either solvent. However, since this work was completed the adenine and hypoxanthine samples used have been examined by the paper electrophoresis technique of Markham & Smith (1951). While the adenine had no detectable impurities, the hypoxanthine preparation was shown to contain more than 50 % of adenine.

Treatments. Water-soluble compounds were applied in aqueous solution. Other compounds were applied as a suspension in water or were brought into solution by heating below 100° in 0.1 and 0.3 % NaHCO₃ or in dilute hydro-

chloric acid solution. (In Matthews 1951 the concentration of NaHCO_3 was incorrectly stated to be 1.0 % instead of 0.1 %.) All percentages of solids in solution are given on a w/v basis. In some experiments compounds were watered on the soil around the base of the plant, but in the majority they were sprayed evenly over the surface of all leaves, using a small hand atomizer.

Measurement of activity of compounds. Activity of compounds was assayed by counts of local lesions, by the number of plants developing systemic symptoms, and by the mean number of days taken to develop systemic symptoms. In most experiments 6–12 plants were used for each treatment. The plants showing systemic symptoms were counted daily; counts made more frequently would increase the subjective error involved in determining when a plant first shows symptoms. Experiments were usually carried on for several weeks until the plants became mature or pot bound. Daily observations of the number of systemically infected plants provided grouped data where the group interval was rather large in relation to the mean (at least for untreated plants). For this reason uniformity trials have so far failed to yield useful information on the form of the distribution of time taken for systemic symptoms to develop. Thus no test for the significance of differences in the data on systemic infections can be made at present. To give an indication of the variation, in a uniformity trial in which fifteen groups of six untreated plants were inoculated with lucerne mosaic virus at dilutions from 1/1 to 1/10,000, the mean number of days for systemic symptoms to appear in groups of six plants varied from 5.8 to 9.0.

RESULTS

Lucerne mosaic virus

Of the nine substituted purines tested only the triazolopyrimidine analogues of guanine, adenine and hypoxanthine had any effect on the development of lucerne mosaic virus infections. Table 1 summarizes a comparative test with these three compounds. Guanazolo was the most effective, except in beans, where only the hypoxanthine analogue reduced the number of local lesions. However, guanazolo at 0.01M caused a marked reduction in the size of the local lesions in beans compared with controls or the other treatments. In tobacco and *N. glutinosa* the adenine analogue at 0.01M caused general stunting involving a virtual cessation of growth in the youngest apical leaves, accompanied by yellowing and some distortion and down-curling of leaves with some necrosis. Such damage began to appear 2–3 days after the first treatment, and plants took several weeks to regain normal growth after treatments ceased. The hypoxanthine analogue had no visible effect on plant growth. At 0.02M guanazolo caused fairly marked stunting of plants and slight yellowing of leaves in *N. glutinosa* and tobacco. At 0.01M this effect was much less severe, and below 0.005M there was usually no detectable effect on growth. When growing conditions were good the damage produced by guanazolo up to about 0.01M disappeared after a few days. Pl. 1, fig. 3,

illustrates the effect of the three analogues on growth of *N. glutinosa*. In bean plants guanazolo caused more severe yellowing and stunting.

Table 1. *Effect of guanine, adenine and hypoxanthine analogues on lucerne mosaic virus in Nicotiana glutinosa, tobacco and beans*

Treatment	In tobacco			In <i>N. glutinosa</i>			In beans
	No. of local lesions, (mean of 12 leaves)	No. of plants out of 6 systemically infected after 25 days	Mean no. of days for systemically-infected plants to show symptoms	No. of local lesions, (mean of 18 leaves)	No. of plants out of 6 systemically-infected after 25 days	Mean no. of days for systemically-infected plants to show symptoms	No. of local lesions, (mean of 24 leaves)
Control A	174	6	5.0	17.0	5	7.2	68
Control B	138	6	5.3	16.5	5	8.1	142
Control C	132	6	5.0	9.7	4	14.2	157
Guanazolo							
0.01 M	2.4	0	—	0.28	0	—	113
0.003 M	5.6	2	14.5	0.56	0	—	135
0.001 M	13.6	6	10.3	4.0	1	17	94
0.0003 M	88	6	6.5	3.3	2	13.5	110
0.0001 M	159	6	5.5	15.5	4	12.7	89
Hypoxanthine analogue							
0.01 M	70	6	6.0	7.4	2	9.5	18.5
0.003 M	37	6	6.3	8.3	4	8.5	73
0.001 M	31	6	6.2	7.9	5	10.4	137
0.0003 M	91	6	5.3	14.7	5	9.6	160
0.0001 M	115	6	5.2	16.1	5	8.4	142
Adenine analogue							
0.01 M	—	0	—	—	5	13.6	—
0.003 M	3.2	6	10.7	3.3	6	8.8	89
0.001 M	11	6	8.2	8.1	6	8.8	120
0.0003 M	48	6	5.3	11.0	6	8.3	75
0.0001 M	122	6	5.3	18.5	5	7.8	150

Compounds. Guanazolo and the adenine analogue at 0.01 M were in solution in 0.1% NaHCO_3 , the other concentrations being prepared by dilution from this. Hypoxanthine analogue was in solution in water.

Times of treatment. Two sprays in the week before and two in the week after inoculation.

Source of inoculum. Sap from infected tobacco diluted 1/100 for *N. glutinosa* and tobacco and 1/80 for beans.

Plant damage. Adenine analogue at 0.01 M caused considerable necrotic damage on inoculated leaves and marked general stunting. Accurate local lesion counts were not possible at this concentration.

At concentrations producing damage inoculation of several leaves after spray treatments increased the damage effects in non-inoculated leaves. This suggests that the wounding produced by carborundum inoculation allows a greater amount of the compound to enter the plant. To test whether guanazolo masked symptoms or prevented systemic movement of active virus, inoculation tests were made after several weeks from the symptomless treated plants in a number of experiments. In all cases local lesions were produced in tobacco only from plants showing systemic symptoms. In some experiments systemic symptoms were less severe in treated plants than in untreated,

and acute early necrosis was absent. However, in one case a test for relative concentration of virus (measured by the number of local lesions produced in tobacco at three dilutions) showed no significant difference between treated and untreated systemically infected plants 30 days after inoculation. In one experiment none of six plants sprayed before and after inoculation developed any local or systemic symptoms; some inoculated leaves showed a few indefinite yellowish areas. Twenty-one days after inoculation tests for the presence of active virus in these six plants were made by inoculation to tobacco. For each plant, inoculations were made from young leaves and from inoculated leaves. Virus could not be detected in any of the young leaves, but the tests from inoculated leaves produced local lesions in three of the six plants. Since the viability *in vitro* of lucerne mosaic virus is about 2-3 days, it is unlikely that these lesions were caused by virus in the initial inoculum remaining on the surface of the leaf. It appears that the virus had multiplied locally to a limited extent without producing definite local lesions and without becoming systemic.

In most experiments guanazolo treatments considerably decreased the number of local lesions. In some the lesions produced in treated plants were all of the same type as those of the controls (i.e. etched rings or solid necrotic spots or a mixture of these types). In other experiments a variable proportion of the local lesions produced in treated plants were much less necrotic than in the controls and graded into vague yellowish spots, which were usually few compared with necrotic or etched lesions. In counting local lesions all lesions which showed any degree of etching or necrosis were included. In addition to decreasing the number of visible local lesions, guanazolo sometimes delayed their appearance for several days.

Guanazolo was more effective in solution in NaHCO_3 than in suspension, and was usually more effective when applied before inoculation than after inoculation. It was ineffective if applied more than 1-2 days after inoculation (Table 2). In general, guanazolo was more effective with more dilute inoculum. Leaf spraying was more effective than watering on the soil, at least when the virus is introduced by mechanical means (Table 3). Pl. 1, figs. 1 and 2, illustrate the effect of guanazolo treatment on lucerne mosaic infections.

Satisfactory levels of infections in control plants were not obtained when lucerne mosaic virus was transmitted to *N. glutinosa* and tobacco by aphids. However, in one experiment, using subterranean clover, spraying with guanazolo was ineffective against aphid-transmitted virus; there was a suggestion that watering with the compound may have had some effect.

In a number of experiments in which infective sap was incubated with 1.0 and 0.1% guanazolo in suspension or in solution in NaHCO_3 for 2 hr., and in which the leaves of test tobacco plants were either washed or left unwashed after inoculation, a significant effect on numbers of local lesions was not found. In these experiments the amount of guanazolo entering the leaf from the inoculum must have been insufficient to affect the number of local lesions.

A number of naturally occurring purines and two pyrimidines were tested

for ability to reverse the virus inhibitory activity of guanazolo. In such tests plants received two sprays with guanazolo and two sprays with the compound under test in the 4 days before inoculation, followed by two or three treatments with the test compound in the week after inoculation. The adenine, guanine and hypoxanthine preparations used annulled the activity

Table 2. *Effect of interval between treatment with guanazolo and time of inoculation. Lucerne mosaic virus in tobacco*

Days of treatments in relation to day of inoculation (day 0)	No. of local lesions, mean of 12 leaves	No. of plants infected out of 6 after 18 days	Mean no. of days for systemically infected plants to show symptoms
Untreated control	166	6	7.8
-7, -6	49	5	12.0
-6, -5	45	5	13.2
-5, -4	44	5	13.4
-4, -3	38	4	14.2
-3, -2	44	4	12.7
-2, -1	34	6	14.5
-1, 0	34	2	10.0
0, 1	57	2	14.5
1, 2	71	5	10.6
2, 3	139	5	8.8
3, 4	145	6	9.5
4, 5	94	6	7.5
5, 6	202	6	7.0
6, 7	181	6	7.5
7, 8	178	6	7.2

Compound. Guanazolo 0.1 % in solution in 0.1 % NaHCO_3 (0.1 % \approx 0.007 M).

Times of treatment. One spray on each of two consecutive days as noted in table.

Source of inoculum. Sap from infected *N. glutinosa* diluted 1/5.

of guanazolo, and xanthine, uric acid, theobromine, theophylline, caffeine, uracil and thymine had no effect. Guanine applied in aqueous suspension had no annulling activity, but had when applied as 0.1 % or 0.01 M solution in dilute hydrochloric acid. However, a valid comparison cannot be made between guanine and the other active compounds because of the plant damage produced by the acid solution. Table 4 summarizes a reversal experiment with adenine. The hypoxanthine preparation (which was later shown to contain between 50 and 60 % of adenine) was more effective than adenine in annulling the activity of guanazolo. In some experiments this mixture increased the number of local lesions and rate of systemic spread above that of unsprayed controls. In view of this greater activity it is possible that hypoxanthine itself has some annulling activity.

Of the three uracil analogues, tested at 0.01 M, only thiouracil had any effect. This compound caused marked yellowing of young leaves, curling down of leaf margins and virtual cessation of apical growth in *N. glutinosa* and tobacco (Pl. 1, fig. 4). This damage may well account, at least in part, for the slight delay which occurred in the development of systemic symptoms of lucerne mosaic.

Table 3. *Application of guanazolo by watering on soil and spraying on leaves. Effect on lucerne mosaic virus in Nicotiana glutinosa*

Time of treatment	Method of treatment	No. of plants systemically infected after 25 days (out of 12 for controls, out of 6 for treatments)	Mean no. of days for systemically infected plants to show symptoms
	Inoculum at 1/1		
Before inoculation	Control untreated	10	6.0
	Sprayed	1	12.0
	Watered	5	7.0
	Sprayed and watered	0	—
After inoculation	Sprayed	4	9.5
	Watered	6	9.2
	Sprayed and watered	5	11.2
Before and after inoculation	Sprayed	0	—
	Watered	5	9.8
	Sprayed and watered	0	—
	Inoculum at 1/10		
Before inoculation	Control untreated	10	7.6
	Sprayed	0	—
	Watered	3	8.3
	Sprayed and watered	0	—
After inoculation	Sprayed	0	—
	Watered	5	9.8
	Sprayed and watered	2	12.5
Before and after inoculation	Sprayed	0	—
	Watered	1	21.0
	Sprayed and watered	0	—

Compound. Guanazolo 0.1 % in solution in 0.1 % NaHCO₃.

Times of treatment. Before inoculation; two treatments, one on each of the two days before inoculation. After inoculation; five treatments at the rate of three per week beginning the day after inoculation. Before and after inoculation; a combination of the above.

Source of inoculum. Infected *N. glutinosa*.

Table 4. *Effect of varying concentrations of adenine on the inhibitory activity of guanazolo. Lucerne mosaic in Nicotiana glutinosa*

Treatment	No. of local lesions (mean of 24 leaves)	No. of plants out of 6 systemically infected after 24 days	Mean no. of days for plants systemically infected to show symptoms
Control	10	0	8.8
Guanazolo	2.6	0	—
Adenine 0.05 M	5.2	6	8.8
Adenine 0.05 M + guanazolo	2.7	3	11.7
Adenine 0.015 M + guanazolo	5.4	5	9.8
Adenine 0.005 M + guanazolo	5.3	5	11.6
Adenine 0.0015 M + guanazolo	4.2	2	14.0
Adenine 0.0005 M + guanazolo	3.8	1	11.0

Compounds. Guanazolo; 0.005 M in solution in 0.1 % NaHCO₃. Adenine; in solution in water.

Times of treatment. Guanazolo on the 4th and 2nd days before inoculation. Adenine on the 3rd and 1st days before, and 3 days in the week after inoculation.

Source of inoculum. Sap from infected *N. glutinosa* diluted 1/2.

Other viruses

The substituted purines tested against lucerne mosaic virus had slight or negligible effects on spotted wilt virus in tomato, potato viruses *X* and *Y* in potato and tobacco and pea mosaic virus in peas (2-aza adenine and the uracil analogues were not tested against these viruses). However, with cucumber mosaic virus more encouraging results have been obtained.

Of nine substituted purines and three analogues of uracil tested guanazolo was the only compound which substantially inhibited the systemic development of cucumber mosaic virus in cucumbers. Treatment with the adenine analogue at 0.01M caused marked stunting, yellowing and some distortion of leaves. New young growth began to appear 7–14 days after treatments. 2:6-diaminopurine caused almost complete cessation of growth, plants becoming very dark green and remaining almost the same size for many weeks after treatments stopped. Plants treated with thiouracil became stunted and yellowed after a few days. The delaying effects produced by 2:6-diaminopurine and the adenine analogue on the appearance of virus symptoms were probably due, at least in part, to the marked effects these compounds had on plant growth. Treatments with 0.02M-guanazolo caused slight stunting, but in about a week plants grew away from this and made as good growth as untreated controls.

When the cucumber mosaic virus was introduced by mechanical inoculation, guanazolo sprayed on the leaves gave complete or almost complete control, but had no effect when watered on the soil. When the virus was introduced by *Myzus persicae* in controlled feeding experiments the compound appeared to have some effect when sprayed on the leaves or watered on the soil.

In other experiments treated and untreated cucumber plants, grown in seedling boxes, were exposed to infection by winged aphids. Boxes of plants were placed in randomized positions a short distance from a group of infected cucumber plants on which winged aphids were placed. In an experiment in which *Hyperomyzus lactucae* was the main aphid employed 69/95 control plants were infected after 48 days while 13/51 plants sprayed with 0.01M guanazolo and 4/42 plants watered with guanazolo were infected. However, in another experiment in which large numbers of winged *Myzus persicae* were employed guanazolo had a much smaller effect (44/47 infected controls; 33/48 infections in plants watered with 0.01M-guanazolo).

DISCUSSION

As methods for application of compounds, leaf spraying and soil watering have the following advantages over the detached leaf, leaf impregnation and tissue culture methods used by previous workers: (i) they are simple to operate; (ii) plant damage can be readily assessed; (iii) any successful results can be immediately tested on a field scale by the same methods; (iv) there is a minimum of interference with the whole growing plant. Disadvantages are: (i) comparatively large amounts of compounds are required for adequate testing; (ii) little is known about the absorption of compounds through leaves

or roots; (iii) accurate control of conditions such as temperature and light is difficult.

The experiments described above show that guanazolo can inhibit the systemic invasion by lucerne mosaic virus of tobacco and *Nicotiana glutinosa*. That this inhibition occurs within the plant and is not merely an inactivation of the virus on the leaf surface is shown by the following: (i) the compound had some effect when watered on the soil; (ii) incubation of the virus in infective sap failed to cause any detectable decrease in infectivity of the virus; (iii) applications of the compound beginning 24 hr. after inoculation had some effect.

It seems likely that the compound has most or all of its effect in the inoculated leaves. In actively growing plants in which the virus became established systemically the compound did not prevent full systemic spread of the virus. The results of inoculation tests from young leaves of symptomless plants did not give any evidence that guanazolo masks systemic symptoms. For control of virus diseases it would usually not matter if a plant became infected at the point of entry, so long as the virus did not become systemic. For this reason, and because of the dubious value of local lesion counts as a quantitative measure, in judging the activities of compounds most weight has been placed on the effects on the systemic development of the virus.

Guanazolo probably has at least three effects in inoculated leaves. First, where all the local lesions in treated and untreated plants were of the same necrotic type, there was almost certainly a decrease in the number of successful entry points. Secondly, the delay in the appearance of local lesions (even of the necrotic type) suggests a delay in multiplication of virus. Thirdly, there was a variable degree of masking of local lesions; for example, it was shown once that the virus had entered and multiplied locally in tobacco without producing any typical lesions.

Why the virus should infect and multiply locally in treated plants without subsequently becoming systemic is not clear. The fewer successful entry points in an inoculated leaf the more slowly the virus will move systemically. However, this effect is small compared with that produced by guanazolo. Guanazolo might delay the multiplication of virus inoculated into a cell, without necessarily limiting the final amount produced. This delay would probably mean a longer period before neighbouring cells became infected, and in these cells multiplication might again be delayed. Cell-by-cell movement of virus in the inoculated leaf might be delayed sufficiently for natural senescent changes in the leaf to occur and prevent systemic movement of the virus.

When sprayed on leaves guanazolo is probably most concentrated in the superficial cells of the inoculated leaf where mechanical damage allows increased amounts of the compound to enter. When virus does move out of the inoculated leaf, particles will be transported to cells in many parts of the plant where the compound is too dilute to be effective.

With mechanically transmitted cucumber mosaic virus spraying with 0.01 M-guanazolo gave complete or almost complete control with negligible effects on plant growth. Watering had no effect on development of infection

in mechanically inoculated plants, but had some effect when the virus was introduced by aphids. When the compound is absorbed through the roots it is probably in highest concentration in the vascular tissue at least for a time. This effect may account for the activity of watered guanazolo when the virus is transmitted by aphids.

Guanazolo inhibits growth of a variety of biological systems. Thus Roblin, Lampen, English, Cole & Vaughan (1945) found that the triazolo analogues of guanine, adenine and hypoxanthine inhibited growth of *Bacterium coli* and *Staphylococcus aureus*. Kidder & Dewey (1949) found guanazolo to be a potent inhibitor of growth of the micro-organism *Tetrahymena geleii*, and Kidder *et al.* (1949) found it inhibited the growth of malignant cells in mice. Growth inhibition by guanazolo has also been shown for virus tumours in *Rumex* (Nickell *et al.* 1950), for fungi (Fries & Panders, 1951) and for algae (Arnow, Sampath, Oleson & Williams, 1952).

For a number of the above systems it has been suggested that guanazolo interferes with the incorporation of guanine into nucleic acids. Similarly, the evidence so far obtained with lucerne mosaic virus suggests that guanazolo may act by blocking reactions involved in the incorporation of guanine and perhaps adenine into the virus nucleic acids.

I wish to thank Mr G. C. Ward, Applied Mathematics Laboratory, Department of Scientific and Industrial Research, for the statistical analyses, Mr S. A. Rumsey for the photographs in Pl. 1, the Lederle Laboratories Division, American Cyanamid Co. for supplies of the triazolo pyrimidines, and Merck and Co. for a sample of 2-aza adenine.

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EXPLANATION OF PLATE

- Fig. 1. Effect of guanazolo on the production of local lesions by lucerne mosaic virus in tobacco. Left: control untreated. Right: sprayed 0.1% guanazolo in solution, treatments commencing the day after inoculation.
- Fig. 2. Effect of guanazolo on systemic development of lucerne mosaic virus in *Nicotiana glutinosa*. Right: untreated control. Left: treated, two sprays 0.1% guanazolo in solution before inoculation.
- Fig. 3. Effect of compounds on growth of *N. glutinosa*. Compounds at 0.01M in solution. Two spray treatments. Plants inoculated but no virus symptoms yet developed. From left to right: (i) control unsprayed, (ii) hypoxanthine analogue, (iii) guanazolo, (iv) adenine analogue.
- Fig. 4. Effect of compounds on growth of tobacco plants. Uninoculated plants. Compounds at 0.01M in solution. From left to right: (i) unsprayed control, (ii) guanazolo, (iii) thiouracil, (iv) methylthiouracil.

(Received 7 November 1952)



R. E. F. MATTHEWS—CHEMOTHERAPY AND PLANT VIRUSES. PLATE 1

DANIELS, G. (1953). *J. gen. Microbiol.* 8, 289-294.

The Digestion of Human Hair Keratin by *Microsporium canis* Bodin

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SUMMARY: *Microsporium canis* Bodin, the causative agent of animal ringworm in children and adults, is able to digest human hair keratin *in vitro*. The process of degradation has been followed by microscopic observation and an analysis of the resulting amino-acids which accumulate in the medium after growth on human hair has been made by chromatographic techniques.

In this investigation attempts have been made to equate the growth of *Microsporium canis* Bodin on human hair with the digestion of hair keratin by the fungus. For this purpose microscopic observations of hair digestion were combined with chromatographic analyses of products which accumulate after growth of the fungus in media containing human hair. The dermatophytes are a specialized group of fungi which cause infections of the skin and dermal appendages, tissues composed principally of keratin. Although the chemical stability of keratins makes them appear to be unfavourable sources of nitrogen for living organisms, keratin digestion has been demonstrated for several organisms, notably the clothes moth (Linderstrøm-Lang & Duspiva, 1935), the goshawk and vulture (Stanković, Arnovljević & Matavulj, 1929), and in certain Actinomycetes (Jensen, 1930). Karling (1948) showed that certain chytrids appear to be limited to growth in keratinized tissues. The ability of ringworm fungi to grow *in vitro* on such tissues has long been known. Thus Bonar & Dryer (1932), and Rogers, Hirschmann & Humfeld (1940) cultivated various dermatophytes on wool; Macfadyen (1894), Nannizzi (1926), and Tate (1929*a*) on feathers; Roberts (1894), Bonar & Dryer (1932), and Williams (1934*a, b*, 1935) on human and other animal hair. Growth on such complex substrata as hair, feathers and nail cannot be considered conclusive proof of the keratinophilic nature of ringworm fungi in view of the associated compounds which occur with the keratin in these materials.

When cultivating twenty-five strains of *Ctenomyces*, *Sabouraudites*, *Trichophyton* and *Epidermophyton* on single human hairs, Vanbreuseghem (1949) observed two types of hair lesion: (1) the hair was eroded over its surface as, for example, with *Trichophyton rubrum*; (2) fissures perpendicular to the long axis of the hair were caused by the intrusion of specialized perforating hyphae, the latter type of lesion frequently resulting in breakage of the hair shaft. By microscopic observations Page (1950) saw the disappearance of particles of finger nail and cow horn, presumably by digestion by *Microsporium gypseum*.

Nannizzi (1926) suggested that ringworm fungi could hydrolyse keratin; Tate (1929*b*), using acetone powder preparations from dermatophytes, failed

to obtain an enzyme preparation which could hydrolyse keratin and no such enzyme has yet been isolated. Keratins are resistant to attack by proteolytic enzymes, and it may be that keratin digestion is not due to enzymic hydrolysis alone. Linderström-Lang & Duspiva (1935) showed in the larva of the clothes moth (*Tineola biselliella* Humm.) the occurrence of a proteolytic enzyme which, over an alkaline range of pH values, readily digested a reduction product of keratin, and that thiol compounds secreted into the gut of the larva were responsible for the reduction of the keratin. Nickerson (1947) suggested that the enzymes produced by the dermatophytes may be able to act only on a reduced form of keratin as do those of the clothes-moth larva.

MATERIALS AND METHODS

Human hair clippings were washed for 24 hr. in running tap water and then extracted with ether on three occasions of 2 hr. each to remove fat. Two experiments were designed: (i) in which the culture medium consisted of human hair + glucose + a simple non-nitrogenous inorganic salt solution; (ii) in which the medium consisted only of human hair + distilled water.

Exp. 1. The culture medium was prepared as follows: six 250 ml. Erlenmeyer flasks each contained 2 g. human hair and the following solution: glucose, 0.6 g.; potassium dihydrogen sulphate cryst., 0.0045 g.; magnesium sulphate cryst., 0.0022 g.; 30.0 ml. distilled water. Three control flasks each contained the above solution without hair, and were sterilized by steaming for 30 min. on three consecutive days. In the case of the six flasks containing hair, the latter was sterilized first, washed with sterile distilled water and the sterile nutrient solution added. Three of the flasks containing hair were inoculated with approximately equal amounts of hyphae of *Microsporum canis* Bodin; the three remaining flasks containing hair were not inoculated and were used as controls, together with those containing only nutrient solution and which were inoculated.

Exp. 2 was a replica of *Exp. 1* except that the glucose + mineral salt solution was replaced by distilled water.

All the flasks were incubated at 20° for 20 days. After this time considerable growth had taken place in the experimental flasks, while no growth had occurred in the control flasks containing only glucose + mineral salt solution or distilled water. The mycelium was in a healthy condition and showed no signs of collapse as observed in stale cultures. Individual hairs were removed from the experimental flasks and from uninoculated control flasks of both experiments and mounted in cotton blue lactophenol for microscopic examination.

The culture fluids were filtered through Seitz filters with sterilizing pads and the filtrates evaporated to dryness under reduced pressure, at a temperature of 25°. The residues were then re-dissolved in distilled water (usually 0.05 ml. or less) and samples taken for chromatographic analysis. Usually 20 μ l. was used for one-dimensional chromatography and this volume also yielded satisfactory two-dimensional chromatograms. The paper partition chromatographic techniques employed were those first described by Consden,

Gordon & Martin (1944). Phenol was used as the first developing solvent and the butanol+acetic acid mixture of Partridge (1948) as the second. Chromatograms were dried at 100° and sprayed with a solution of 0.1 g. ninhydrin in 100 ml. *n*-butanol saturated with water, for the detection of amino-acids. No attempt was made to separate the leucine isomers.

RESULTS

Microscopic observations

Hairs removed from control flasks and mounted in cotton blue lactophenol remained unstained and showed no signs of degradation; the cells of the cuticle showed no tendency to scale off or separate from the hair surface (Pl. 1, fig. 1). In contrast, hairs removed from experimental flasks and similarly mounted showed varying degrees of digestion. An early stage in attack is shown in Pl. 1, fig. 2; a germinating microconidium has given rise to a short hypha which has extended for some distance along the surface of the hair before penetrating and lifting up a small group of cuticular cells, together with a small number of underlying cells of the cortex. The actual method of hyphal penetration has not been established. In Pl. 1, fig. 3, stout much-branched hyphae extend along the longitudinal axis of the hair and are closely applied to the surface of the hair. Some of the finer branches terminate in minute much-branched hyphae around which localized areas of the hair take up intense stain. These may represent surface views of proliferating hyphae beneath the cuticle after initial penetration has been effected. Having gained access to the hair a process of hyphal proliferation takes place beneath the cuticle, resulting in the formation of aggregations of hyphae comprised of short much-branched segments. The pressure on the cuticle produced by the underlying aggregations of hyphae results in extensive rupture of the cuticle. The fate of the cuticle beyond this stage was difficult to ascertain. It is possible that digestion takes place *in situ*, although isolated cuticular cells have been observed in the medium which indicated that these cells may be sloughed off the disintegrating surface of the hair shaft. The aggregations of hyphae digest their way into the cortex of the hair forming spreading lesions of the type observed in Pl. 1, figs. 4*a*, *b*.

From these lesions longitudinal extension of hyphae takes place, so that these frequently connect with hyphae from other lesions, resulting in degradation of the hair until they present appearances shown in Pl. 1, figs. 5 and 6, and Pl. 2, figs. 7–9. In some instances stout hyphae, designated by Vanbreuseghem (1949) as organs of perforation, are produced by the fungus, and these make their way into the cortex of the hair. Organs of this type were formed in two ways; (1) beneath nodules or aggregations of hyphae as a result of division and inward growth of certain segments; (2) by an inward growth of individual hyphae. They are composed of from two to ten cells (Pl. 2, fig. 10*a*, *b*). In some cases the fissures produced by perforating organs passed straight through the hair shaft, although in the main they extended only as far as the medulla. From the perforating organs longitudinal digestion of the hair

shaft takes place, in some cases by the development of lateral branches which connect with lateral branches from other perforating organs (Pl. 2, fig. 11) and in other cases by presumed diffusion of keratin-digesting substances from the cells of the perforating organs. Anastomoses may take place between adjacent cells of perforating organs which enter the hair shaft in pairs (Pl. 2, fig. 12). Morphological features of perforating organs are best studied after clearing the hair in N -NaOH. Continuous digestion of the hair results in the disappearance of large portions of the hair shaft, leaving the original hair in fragments; the extent of this fragmentation depends on the severity of the fungal attack. The intensity with which the hairs became stained with cotton blue-lactophenol depended on the extent of hair degradation.

Chromatographic analyses

One-dimensional chromatograms indicated the presence of abundant ninhydrin-reacting substances in the culture filtrates from the experimental flasks containing hair + glucose + salt solution and their absence from both control solutions, showing that these substances had been produced during the growth of the fungus on the hair. Two-dimensional chromatograms of the filtrates from experimental flasks before and after hydrolysis with $6N$ -HCl for 24 hr. at 100° were run and compared with two-dimensional chromatograms of human hair hydrolysate (prepared by heating human hair with $6N$ -HCl in sealed tubes for 24 hr. at 100°). In the chromatograms of the culture filtrate material and human hair hydrolysate the following amino-acids were identified: aspartic acid, glutamic acid, glycine, alanine, threonine, histidine, arginine, lysine, proline, tyrosine, leucine, phenylalanine, valine, serine and methionine. The identification of the spot produced by methionine was confirmed by treatment of the solution with hydrogen peroxide; in subsequent chromatograms the methionine spot had disappeared and a new spot appeared in the position of methionine sulphone. Cystine appeared in the form of cysteic acid after oxidation with hydrogen peroxide. Similar results were obtained from the filtrates of the hair + distilled water cultures except that phenylalanine, proline, tyrosine and methionine were not detected.

DISCUSSION

Microscopic observations of *Microsporum canis* growing on human hair are of value in investigating the ability of the fungus to digest human hair keratin. Vanbreuseghem (1949, 1950) described a diagnostic method, based upon the cultivation of dermatophytes on individual hairs, for the digestion of hair keratin by ringworm fungi. Visual criteria for keratin digestion give no indication of the nature of the chemical processes involved. It has been shown by chromatographic techniques in the present work that amino-acids accumulate in cultures containing human hair as sole source of nitrogen, after growth of *M. canis* for 20 days at 20° . The ultimate source of these amino-acids is the keratin of the hair, and it is presumed that they are produced by the digestion of hair keratin by the fungus. Evidence for this assumption is afforded by

the similarity of the mixture of amino-acids which occurs in the media after growth of the fungus with that obtained by the hydrolysis of human hair with 6N-HCl.

After growth of *Microsporium canis* on human hair in glucose + mineral salt solution, all the amino-acids occurring in human hair keratin can be detected in the medium, while after growth on human hair + distilled water, proline, tyrosine, methionine and phenylalanine were not detected. It may be that complete degradation of hair keratin occurs when a nutrient solution of glucose and non-nitrogenous mineral salts enriches the medium, and that incomplete breakdown occurs when distilled water only is added to the hair. A second explanation would be that complete degradation of the hair takes place in both cases and that complete utilization of the four amino-acids which were not detected occurs when the fungus is grown on hair + distilled water, while in glucose + mineral salt medium the four amino-acids accumulate since they are not further metabolized. Or perhaps the four amino-acids may be present in the experimental filtrates from hair + distilled water in insufficient quantities for detection in chromatograms by the ninhydrin reaction.

I am indebted to Prof. C. W. Wardlaw for encouragement and advice received during the course of this investigation, to Dr J. Curry for many valuable suggestions, and Dr A. Allsopp for advice on the use of chromatographic techniques. I am indebted to Mr E. Ashby for the photographic illustrations.

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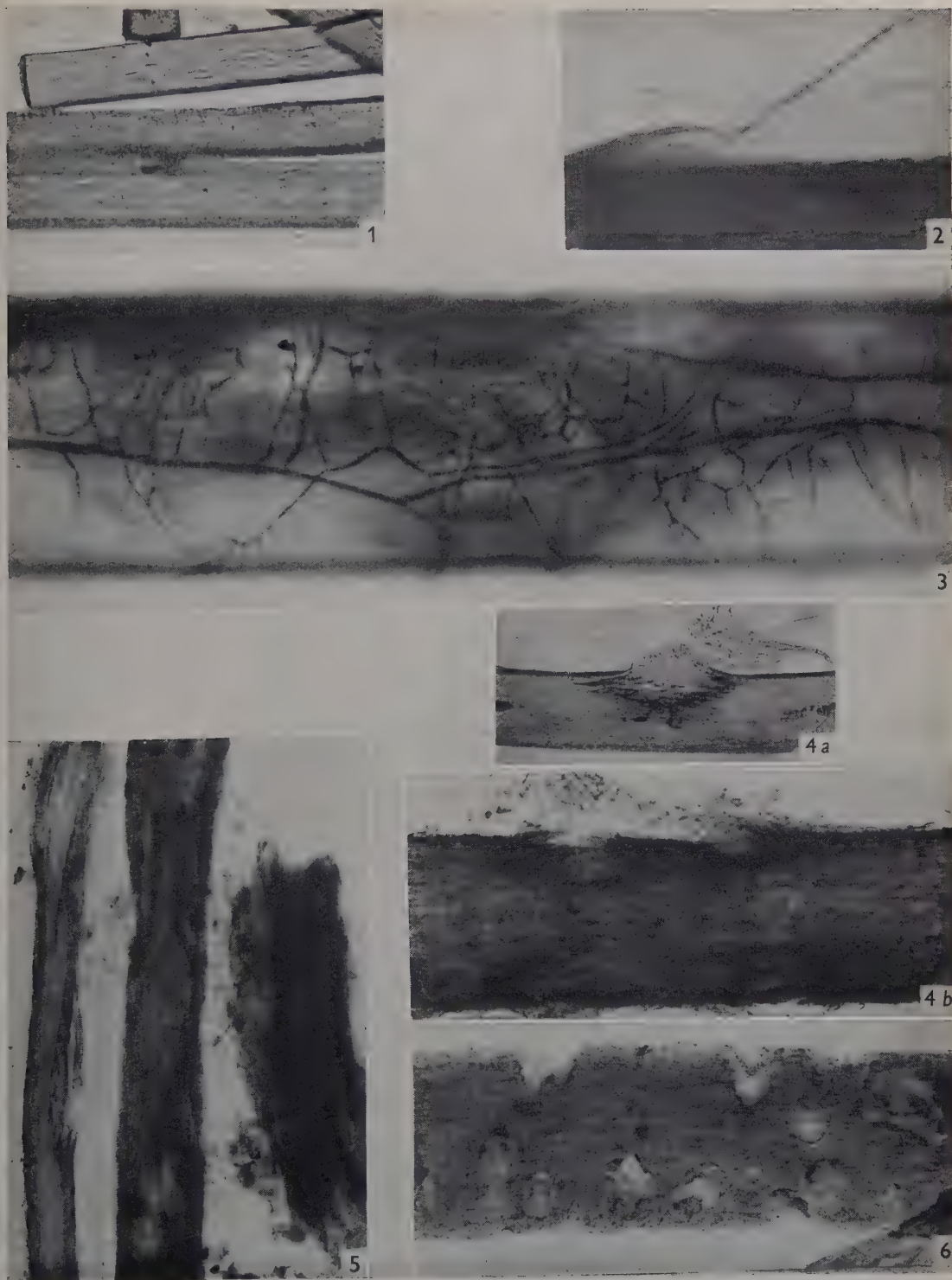
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EXPLANATION OF PLATES

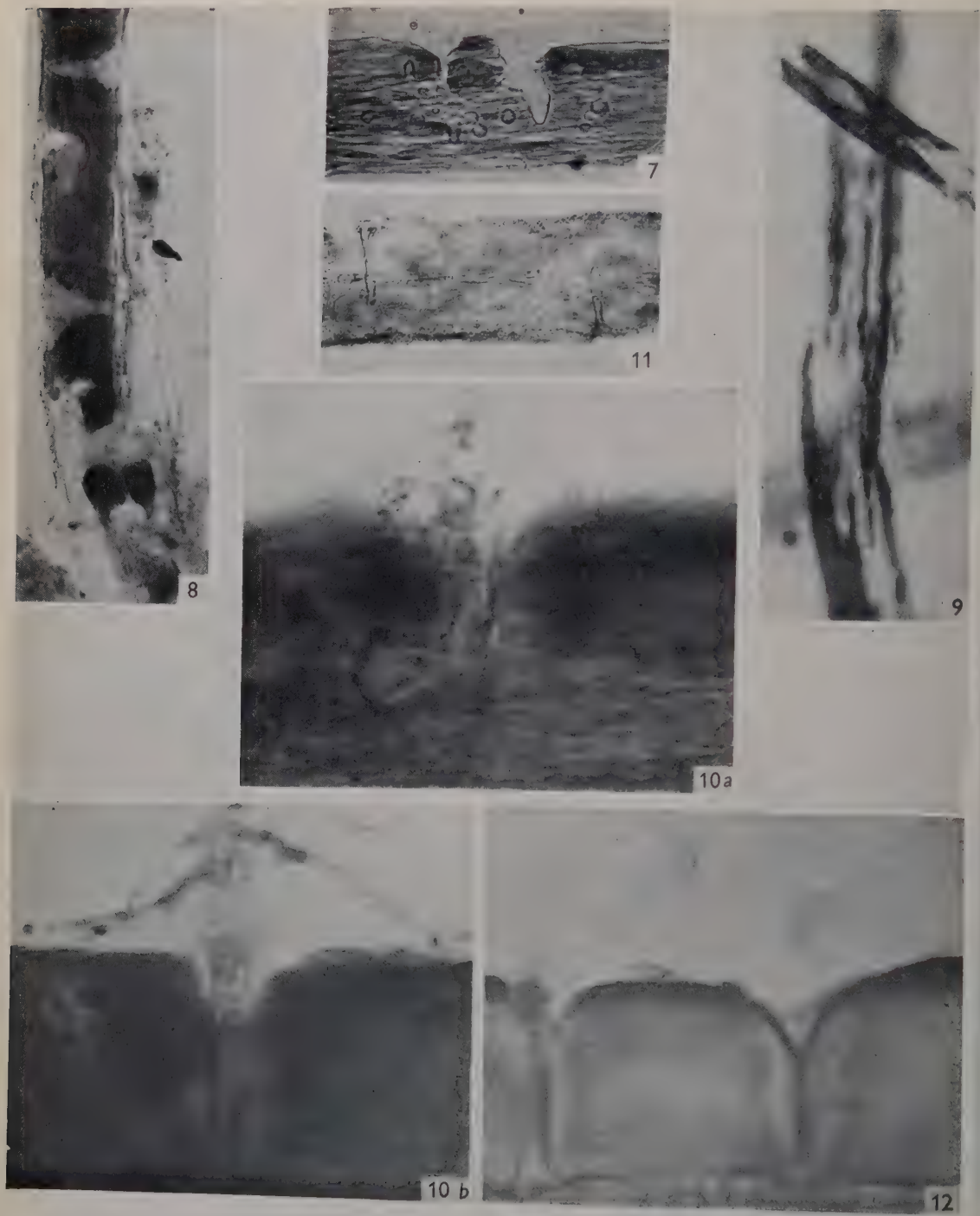
PLATES 1 AND 2

- Fig. 1. Normal, unattacked hairs from a control flask. $\times 110$.
- Figs. 2-9. Stages in the digestion of human hair by *Microsporum canis* Bodin. Fig. 2, $\times 660$; fig. 3, $\times 330$; fig. 4*a*, $\times 55$; fig. 4*b*, $\times 165$; figs. 5, 6, 8 and 9, $\times 110$; fig. 7, $\times 165$.
- Figs. 10*a, b*. Perforating organs of *M. canis* Bodin within the hair shaft. $\times 660$.
- Fig. 11. Perforating organ showing lateral branching within the hair shaft. $\times 165$.
- Fig. 12. Anastomoses between segments of adjacent perforating organs entering the hair shaft in pairs. $\times 660$.

(Received 16 September 1952)



G. DANIELS—DIGESTION OF HAIR BY *MICROSPORUM CANIS*. PLATE 1



G. DANIELS—DIGESTION OF HAIR BY *MICROSPORUM CANIS*. PLATE 2

KLECZKOWSKI, A. (1953). *J. gen. Microbiol.* 8, 295-301.

A Method for Testing Results of Infectivity Tests with Plant Viruses for Compatibility with Hypotheses

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SUMMARY: A statistical method is described to test the compatibility between results of local-lesion counts and hypotheses relating changes in infectivity of plant-virus preparations to treatments applied to them. The method allows the variance-ratio test to be applied: it involves establishing a relation between numbers of lesions and virus concentration for each experiment and a logarithmic transformation of lesion counts to make variance independent of the magnitude of the variate. Its use is illustrated with results obtained to see whether inactivation of viruses by ultraviolet radiation is a first order reaction.

When the infectivity of plant-virus preparations is assayed by the local-lesion method, exact conclusions are difficult to draw because there is no simple relationship between numbers of lesions and virus concentration; also, standard errors of individual numbers are high and depend on the magnitude of the number (Kleczkowski, 1949). With no method to overcome these difficulties, compatibility between experimental results and a hypothesis predicting quantitative changes in infectivity could be judged only by intuition and not tested statistically. The purpose of this paper is to describe a method whereby objective statistical tests can replace subjective judgement.

THE METHOD

Experimental design

When infectivity of several virus preparations is to be assayed, each of them can be inoculated at one convenient dilution for comparison with a standard preparation inoculated at several dilutions. Each inoculum is rubbed on a number of half-leaves (or whole leaves) distributed in such a way as to eliminate as many causes of variation as possible. Thus each inoculum must occur an equal number of times on each of a number of plants, or of blocks of plants, and, if the leaves occupy different positions on the stem, each inoculum must occur an equal number of times on each leaf position or on each leaf level comprising not more than two neighbouring positions. Some convenient distributions are given by Kleczkowski (1950).

The statistical treatment to be described can be applied only if *all* the inocula produce on the average at least about five lesions per half-leaf (or per leaf) and not more than can be conveniently counted.

Statistical test

Let us assume that the hypothesis under test predicts that a given treatment alters the magnitude of virus activity by a factor p which is a function f of a variable v , so that

$$p=f(v, k), \quad (1)$$

where k is an adjustable constant. The variable v can be any quantitative aspect of a treatment, such as time of its duration, concentration of a reagent, etc. There can be more than one function and/or more than one variable or one constant involved, but this does not alter the principle of the procedure.

The procedure is based on two facts. First, it is usually possible, by suitably adjusting the values of three constants, N , ξ and λ , to express the relationship between the numbers of lesions (Y) and the concentration of a virus preparation in the inoculum (x) by the equation

$$Y = \frac{N}{\lambda\sqrt{2\pi}} \int_{-\infty}^u \exp \left\{ -\frac{1}{2} \left(\frac{r-\xi}{\lambda} \right)^2 \right\} dr, \quad (2)$$

where $u = \log_{10} x$ if the virus preparation has not been subjected to the treatment under test or $u = \log_{10} px$ if the preparation has been subjected to the treatment which has altered its activity by the factor p of equation (1). (The value of Y/N can be obtained from a table of 'probits'.)

Secondly, if individual numbers of lesions (y) on each half-leaf (or whole leaf) are transformed by the formula

$$z = \log_{10}(y+c), \quad (3)$$

(where c is a constant whose value can be determined from experimental data or taken as anything between 5 and 15), the values of z are approximately normally distributed with a standard error independent of their magnitude (Kleczkowski, 1949). It is essential that the mean value of y , obtained with any inoculum, should not be smaller than about 5, otherwise the transformation (3) may not make the standard error of z independent of its magnitude.

Thus all the numbers of lesions per half-leaf (or whole leaf) are transformed according to the formula (3) and the values of z thus obtained are used throughout.

An estimate of the variance (s^2) of single values of z is then obtained by means of the customary analysis of variance, when use is made of the experimental design eliminating a number of known sources of variation in mean values of z for each inoculum.

Further procedure resembles that used to test different equations for expressing the relationship between numbers of lesions and virus concentrations (Kleczkowski, 1950).

Mean values of z are decimal logarithms of geometric means of experimental values ($y+c$), whereas the values of Y (given by equation (2)) are expected arithmetic means of the values of y . Thus the values of Y must be transformed into decimal logarithms of expected geometric means of values ($y+c$), and the logarithms can then be directly compared with mean values of z . The transformation is

$$Z = \log_{10}(Y+c)_1 - 1.149s^2, \quad (4)$$

where Z is an estimate of the logarithm of the geometric mean and s^2 the estimate of the variance of single values of z obtained from the analysis of variance (Kleczkowski, 1950).

The values of the constants k , N , ξ and λ of equations (1) and (2) are adjusted by the method of least squares, i.e. by minimizing the value

$\Sigma((\bar{z}_i - Z_i)^2$, where \bar{z}_i 's are the mean values of z obtained with different inocula and Z_i 's are the corresponding values of Z of equation (4). As the values of z can be assumed to be normally distributed around the corresponding values of Z with the same variance, the method of the least squares of adjusting the values of the constants is here equivalent with the method of maximum likelihood.

If each \bar{z} is a mean of n values of z , $n\Sigma(\bar{z}_i - Z_i)^2/W$ (where W is the number of degrees of freedom equal to the number of inocula minus the number of adjustable constants), should give an estimate of the variance of single values of z independently of that given by s^2 , obtained from the customary procedure of the analysis of variance. If the hypothesis on which equation (1) is based is true, the two estimates should be compatible, and this can be tested by the variance-ratio test.

Adjusting the values of the constants is usually the most laborious part of the procedure. There may be several ways of doing this but all start by guessing likely values of the constants, substituting them and computing the results. Successive improvements are then made. Sometimes the two estimates of the variance can be made compatible by a few successive improvements of the values of the constants simply by guesses helped by inspecting the last results. Various graphical methods of adjusting the constants can also be used. If the two estimates of the variance still remain incompatible, several successive improvements of the constants can finally be made by computing increments to be added to the constants by solving the 'normal' equations:

$$\begin{aligned}\Delta N \Sigma \left(\frac{\partial Z_i}{\partial N} \right)^2 + \Delta \xi \Sigma \left(\frac{\partial Z_i}{\partial N} \frac{\partial Z_i}{\partial \xi} \right) + \Delta \lambda \Sigma \left(\frac{\partial Z_i}{\partial N} \frac{\partial Z_i}{\partial \lambda} \right) + \Delta k \Sigma \left(\frac{\partial Z_i}{\partial N} \frac{\partial Z_i}{\partial k} \right) &= \Sigma \left(\frac{\partial Z_i}{\partial N} (\bar{z}_i - Z_i) \right), \\ \Delta N \Sigma \left(\frac{\partial Z_i}{\partial \xi} \frac{\partial Z_i}{\partial N} \right) + \Delta \xi \Sigma \left(\frac{\partial Z_i}{\partial \xi} \right)^2 + \Delta \lambda \Sigma \left(\frac{\partial Z_i}{\partial \xi} \frac{\partial Z_i}{\partial \lambda} \right) + \Delta k \Sigma \left(\frac{\partial Z_i}{\partial \xi} \frac{\partial Z_i}{\partial k} \right) &= \Sigma \left(\frac{\partial Z_i}{\partial \xi} (\bar{z}_i - Z_i) \right), \\ \Delta N \Sigma \left(\frac{\partial Z_i}{\partial \lambda} \frac{\partial Z_i}{\partial N} \right) + \Delta \xi \Sigma \left(\frac{\partial Z_i}{\partial \lambda} \frac{\partial Z_i}{\partial \xi} \right) + \Delta \lambda \Sigma \left(\frac{\partial Z_i}{\partial \lambda} \right)^2 + \Delta k \Sigma \left(\frac{\partial Z_i}{\partial \lambda} \frac{\partial Z_i}{\partial k} \right) &= \Sigma \left(\frac{\partial Z_i}{\partial \lambda} (\bar{z}_i - Z_i) \right), \\ \Delta N \Sigma \left(\frac{\partial Z_i}{\partial k} \frac{\partial Z_i}{\partial N} \right) + \Delta \xi \Sigma \left(\frac{\partial Z_i}{\partial k} \frac{\partial Z_i}{\partial \xi} \right) + \Delta \lambda \Sigma \left(\frac{\partial Z_i}{\partial k} \frac{\partial Z_i}{\partial \lambda} \right) + \Delta k \Sigma \left(\frac{\partial Z_i}{\partial k} \right)^2 &= \Sigma \left(\frac{\partial Z_i}{\partial k} (\bar{z}_i - Z_i) \right).\end{aligned}$$

The partial derivatives are

$$\begin{aligned}\frac{\partial Z}{\partial N} &= \frac{0.4343}{(Y+c)\lambda\sqrt{(2\pi)}} \int_{-\infty}^u \exp \left\{ -\frac{1}{2} \left(\frac{r-\xi}{\lambda} \right)^2 \right\} dr, \\ \frac{\partial Z}{\partial \xi} &= -\frac{0.4343 N}{(Y+c)\lambda\sqrt{(2\pi)}} \exp \left\{ -\frac{1}{2} \left(\frac{u-\xi}{\lambda} \right)^2 \right\}, \\ \frac{\partial Z}{\partial \lambda} &= -\frac{0.4343 N(u-\xi)}{(Y+c)\lambda^2\sqrt{(2\pi)}} \exp \left\{ -\frac{1}{2} \left(\frac{u-\xi}{\lambda} \right)^2 \right\}, \\ \frac{\partial Z}{\partial k} &= \frac{0.1886 N}{(Y+c)p\lambda\sqrt{(2\pi)}} \exp \left\{ -\frac{1}{2} \left(\frac{u-\xi}{\lambda} \right)^2 \right\} \frac{\partial p}{\partial k}.\end{aligned}$$

As soon as the two estimates of the variance of z do not differ significantly, it can be concluded that the hypothesis under test is compatible with the

experimental data. If the two estimates of the variance remain incompatible, the adjustment of the constants can be discontinued when it becomes obvious that the value $\Sigma(\bar{z}_i - Z_i)^2$ cannot be further reduced to any appreciable extent. It can then be concluded that the hypothesis is incompatible with the data.

The course of inactivation of two viruses by ultraviolet radiation

As an example of application of the method results of three experiments made by Bawden & Kleczkowski (1953) with ultraviolet-irradiated viruses (Exps. 1, 2 and 4 in Table 3 of their paper) will be tested for compatibility with the hypothesis that inactivation by ultraviolet radiation is a first-order reaction. If it is, the fraction p of the original activity left after the time t of irradiation will be

$$p = e^{-kt}, \quad (5)$$

where k is an adjustable constant whose value depends on experimental conditions. Equation (1) takes the form of (5), the variable v becoming t .

Table 1 shows the results of analyses of variances carried out on the values of z obtained by (3) using 5 as the constant c , and Tables 2-4 show the results of testing the experimental data for compatibility with the hypothesis.

In Exp. 4 (Table 4) tobacco mosaic virus was irradiated at two different concentrations, so that two different values of k (k_1 and k_2) had to be used. Had it been necessary to use the 'normal' equations for adjusting the values of the five constants involved, there would have been five such equations.

The results of Exps. 1 and 4 (Tables 2 and 4) are compatible with the hypothesis, whereas those of Exp. 2 (Table 3) are not. However, although the deviations of the values of \bar{z} from the corresponding values of Z are statistically significant, they are small, so that it can be concluded that if the inactivation is not exactly a first-order reaction, its course approaches that of a first-order reaction quite closely. It is, of course, also possible that the inactivation is exactly a first-order reaction and that Exp. 4 contained some experimental inaccuracies that were not allowed for in the treatment of the data.

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(Received 19 September 1952)

Table 1. *Analyses of variances*

Virus	Host plant	Exp. 1			Exp. 2			Exp. 4		
		Rothamsted tobacco necrosis virus			Rothamsted tobacco necrosis virus			Tobacco mosaic virus		
		French bean			French bean			<i>Nicotiana glutinosa</i>		
		Degrees of freedom	Sums of squares	Mean square (s^2)	Degrees of freedom	Sums of squares	Mean square (s^2)	Degrees of freedom	Sums of squares	Mean square (s^2)
	Between inocula	7	12.1369	—	7	17.6574	—	11	12.9707	—
	Between leaf levels*	—	—	—	—	—	—	2	1.6568	—
	Between plants (or blocks of plants)	13	0.4990	—	13	0.7670	—	8	5.1102	—
	Left-right half-leaves	1	0.0163	—	1	0.0181	—	—	—	—
	Residual	90	1.4004	0.0156	90	0.9797	0.0109	86	4.4219	0.0514
	Total	111	14.0526		111	19.4222		107	24.1596	

**Nicotiana glutinosa* plants used in Exp. 4 had six leaves each. The six leaf positions were divided into three levels and each inoculum occurred three times in each level.

Table 2. *Comparison of experimental with computed values for Exp. 1*

Rothamsted tobacco necrosis virus irradiated as a 1% solution

Time of irradiation t (min.)	\log_{10} of concentration (in %) at which the virus was inoculated	u	Z	\bar{z}	Expected fraction of the original activity
2.480	-3.0	-3.495	2.1524	2.1314	0.32
4.243		-3.848	2.0017	1.9957	0.14
6.006		-4.200	1.8023	1.8286	0.063
7.769		-4.552	1.5591	1.5071	0.029
0	-3.7	-3.7	2.0713	2.0657	1.00
0	-4.2	-4.2	1.8057	1.8014	
0	-4.7	-4.7	1.4472	1.4729	
0	-5.2	-5.2	1.0620	1.1036	

Constants: $N=260$; $\xi=-3.6$; $\lambda=0.83$; $k=0.46$.

$$\Sigma(\bar{z}_i - Z_i)^2 = 0.006314; n=14; W=4.$$

$$\text{Variance ratio: } R=1.42 \begin{cases} n_1=4. \\ n_2=90. \end{cases}$$

$$P>0.2.$$

Table 3. *Comparison of experimental with computed values for Exp. 2*

Rothamsted tobacco necrosis virus irradiated as a 0.1% solution.

Time of irradiation t (min.)	\log_{10} of concentration (in %) at which the virus was inoculated	u	Z	\bar{z}	Expected fraction of the original activity
0.58	-3.0	-3.736	2.0313	1.9807	0.18
1.00		-4.268	1.6835	1.6221	0.054
1.42		-4.801	1.2903	1.3443	0.016
1.84		-5.333	0.9334	0.9600	0.0047
0	-3.7	-3.7	2.0512	2.0943	1.000
0	-4.2	-4.2	1.7315	1.7714	
0	-4.7	-4.7	1.3650	1.3700	
0	-5.2	-5.2	1.0185	0.9921	

Constants: $N=580$; $\xi=-2.8$; $\lambda=1.03$; $k=2.92$.

$$\Sigma(\bar{z}_i - Z_i)^2 = 0.0141; n=14; W=4.$$

$$\text{Variance ratio: } R=4.5275 \begin{cases} n_1=4. \\ n_2=90. \end{cases}$$

$$P=0.001 \text{ (approximately).}$$

Table 4. Comparison of experimental with computed values for Exp. 4

Tobacco mosaic virus			Z	z	Expected fraction of the original activity
Time of irradiation <i>t</i> (min.)	\log_{10} of concentration (in %) at which the virus was inoculated	<i>u</i>			
Virus irradiated as a 0.5% solution	3.85	-3.002	2.0876	1.9633	0.32
	7.70	-3.503	1.8383	1.8933	0.099
	11.55	-4.005	1.5595	1.5178	0.031
	15.40	-4.506	1.2644	1.3111	0.0098
Virus irradiated as a 0.01% solution	0.575	-3.163	2.0038	1.9333	0.22
	1.150	-3.822	1.6619	1.7800	0.047
	1.725	-4.485	1.2784	1.1989	0.0104
	2.300	-5.147	0.9304	0.9711	0.0023
Unirradiated virus	0	-3.0	2.0876	2.1456	1.000
	0	-3.5	1.8383	1.7689	
	0	-4.0	1.5595	1.5833	
	0	-4.5	1.2697	1.2711	

Constants: $N = 850$; $\xi = -1.6$; $\lambda = 1.4$;
 $k_1 = 0.8$ (for 0.5% virus solution);
 $k_2 = 2.65$ (for 0.01% virus solution).
 $\Sigma(\bar{z}_1 - Z_i)^2 = 0.0589$; $n = 9$; $W = 7$.
Variance ratio: $R = 1.473$ $\begin{cases} n_1 = 7. \\ n_2 = 86. \end{cases}$
 $P = 0.2$ (approximately).

The Influence of Hexose Phosphates, Calcium and Jute Extract on the Formation of Perithecia by *Chaetomium globosum*

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SUMMARY: Chromatographic examination of an aqueous extract of jute showed the presence of inorganic phosphate, glucose-6-phosphate, fructose-1:6-diphosphate and calcium ion. A mixture of the last three in the amounts estimated to be present in the jute extract stimulated early and vigorous formation of perithecia of *Chaetomium globosum* in the same manner as does jute extract, although the amount of vegetative growth was appreciably less. No indication of the presence of a specific 'fruiting factor' was obtained.

The addition of a small amount of an aqueous extract of jute to a Czapek-Dox medium containing 0.5 % glucose was shown by Buston & Basu (1948) to affect the growth and formation of perithecia of *Chaetomium globosum* Kunze, in that vegetative growth became more vigorous and perithecia appeared 2 or more days earlier and in greater numbers than on the basal medium. So far as fruiting is concerned, Basu (1951) showed that a similar effect was produced by calcium within rather narrow limits of concentration (c. 10 p.p.m.), and that calcium was present in jute extract. Buston & King (1951) noted that fruiting was accelerated by small amounts of glucose-1-phosphate or fructose-1:6-diphosphate (cf. Hawker, 1948) and detected in jute extract traces of acid-labile organic phosphate, presumed to be hexose phosphates. They concluded, however, that the effect of jute extract was unlikely to be due entirely to the presence of these compounds, and pointed out that since the activity of the extract was partially destroyed by acid hydrolysis the calcium ion alone could not be responsible for the stimulation of fruiting. They did not investigate the action of calcium in combination with hexose phosphates; this is the subject of the present communication.

EXPERIMENTAL

The strain of *C. globosum* was that used previously in this laboratory, and designated by Basu no. 79. The basal medium contained (per l.): NaNO_3 , 2 g.; KH_2PO_4 , 1 g.; KCl , 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; glucose, 5 g.; agar, 20 g. All salts were Analar (British Drug Houses Ltd.) where available, or were recrystallized before use. The jute extract was prepared as described by Buston & Basu (1948), its concentration being such that 1 ml. was equivalent to 1 g. jute. Sterilization was done at 10 lb./sq.in. for 20 min.; incubation was at 30°.

Using the method described by Buston & King (1951) the combined effect

of calcium and glucose-1-phosphate was examined. The time of appearance of perithecia was noted, and after 96 hr. an estimate of the number of perithecia formed was made by an adaptation of the method of Asthana & Hawker (1936). These authors defined 'perithecial frequency' as the average number of perithecia/unit area of an agar culture, i.e. in a standard microscopic field. In making the counts, the cover-plate of the dish was replaced by a transparent plastic template, divided into ten equal radial sectors, which rested on top of the medium. Usually counts of three fields were made in each sector (30 fields/plate). The results (Table 1) showed that while mycelial growth and fruiting were most vigorous in the presence of jute extract, a similar acceleration of fruiting was produced by the extract and by glucose-1-phosphate, alone or in combination with calcium. Perithecial frequency was markedly increased by calcium, but was always less than that given by jute extract; also, the perithecia formed in presence of jute extract were considerably larger than those formed on the other media.

Table 1. *Effect of glucose-1-phosphate and calcium on growth and sporulation of Chaetomium globosum*

Supplement to basal medium	Time of observation (hr.)				Perithecial frequency at 96 hr.
	48	72	96	120	
	Colony diameter (mm.)				
Ca, 1 p.p.m.	—	13.6	21.1	24.5	—
Ca, 10 p.p.m.	—	11.0	19.5	23.5*	6.5†
G-1-P, 0.05 %	11.6	21.3	25.6*	27.8	4.80
G-1-P 0.05 % + Ca 1 p.p.m.	13.5	21.5	24.9*	28.1	5.50
G-1-P, 0.05 % + Ca 10 p.p.m.	13.5	21.8	26.3*	28.3	6.25
Jute extract	16.8	31.4	46.6*	65	7.88

G-1-P = glucose-1-phosphate. Perithecial frequency = average no. of perithecia per microscopic field (magnification 100 ×).

* First appearance of perithecia.

† At 120 hr.

Chromatographic investigation of jute extract

The extract was examined for sugar phosphates by paper strip chromatography, using as solvent a methanol/formic acid/water mixture (80/15/5, v/v.; Bandurski & Axelrod, 1951) and spraying with the acid molybdate reagent of Hanes & Isherwood (1949). The presence of inorganic phosphate, glucose-6-phosphate and fructose-1:6-diphosphate was established; no glucose-1-phosphate was detected. Calcium was also demonstrated by the method of Pollard, McOmie & Stevens (1951) using a spray of kojic acid and examining the dried ammonia-treated paper in ultraviolet light.

Since paper chromatography had served to separate certain components of jute extract known to be concerned with stimulating the formation of perithecia it seemed possible that further information on the relative effect of the different factors might be obtained by growing the organism directly on the paper strip after running the chromatogram, without applying the spray reagents. Accordingly, a portion of jute extract was concentrated to approxi-

mately 1/5 volume, and 0.13 ml. portions (equivalent to the amount usually incorporated into 15 ml. of medium) were spotted on strips of filter-paper 5 × 30 cm. at 2 cm. from the lower edge; ascending chromatograms were allowed to run in methanol/formic acid/ water at *c.* 2° until the solvent front had just reached the upper edge of the strip. The strips were dried in air overnight at room temperature and cut into 5 cm. lengths, each of which was placed in a Petri dish and covered with 15 ml. of sterile basal medium; after standing for 20 hr. to allow diffusion the plates were inoculated from a young culture of *C. globosum*.

After 72 hr. perithecia were observed in three of the cultures from each strip, whilst the others showed vegetative growth only. Those sections of the strips which gave positive results corresponded to the positions occupied by the two sugar phosphates and by calcium, as shown by a control chromatogram. This result confirmed that calcium, glucose-6-phosphate and fructose-1:6-diphosphate all have a stimulant effect on the formation of perithecia. It appeared very unlikely that any other constituent of jute extract was similarly effective, unless the chromatogram failed to separate it from one of these three.

Attempts at quantitative measurement

Attempts were made to utilize the formation of perithecia as a basis for estimating the amount of each stimulant in jute extract. Pieces of filter-paper 5 cm. square were spotted with known quantities of glucose-6-phosphate, fructose-1:6-diphosphate (potassium salts) and CaCl₂, placed in Petri dishes with 15 ml. sterile medium, allowed to stand for 20 hr., inoculated and incubated as usual. After 72 hr. a perithecial count was made as described above. With a strong light beneath the plate it was easily possible to make the count through the filter-paper; 30 fields/plate were counted. The results are summarized in Table 2. It appeared that for each of the three recognized stimulants there was an optimum concentration, as already observed for calcium by Basu (1951). Filter-paper itself may contain some factor which stimulates formation of perithecia (Basu, 1947). In all the present experiments the paper used (Whatman no. 1) was extracted with methanol/formic acid and dried before use, this treatment having been shown to remove the stimulant noted by Basu.

Table 2. *Effects of hexose phosphates and of calcium on perithecial frequency (P.F.) in 72 hr. cultures of C. globosum*

	Glucose-6-phosphate				
Concn. (%)	0.01	0.02	0.03	0.04	0.05
P.F.	5.2	6.6	8.8	7.6	4.7
	Fructose-1:6-diphosphate				
Concn. (%)	0.004	0.008	0.012	0.016	—
P.F.	6.0	7.2	8.6	5.2	—
	Calcium				
Concn. (p.p.m.)	1	5	10	15	—
P.F.	3.9	4.6	7.6	4.4	—

Further chromatograms were run with the jute extract, and those portions of the strip corresponding to the positions of the three recognized stimulants were tested as described above. Perithecia invariably appeared within 72 hr., while in control experiments their appearance was delayed by some 36–48 hr. Perithecial counts were made after 72 hr., and by comparison with curves based on the figures in Table 2, the approximate concentration of each factor was estimated. The portion containing glucose-6-phosphate gave a perithecial count of 6.4 corresponding approximately to 0.025 % of the ester; that containing fructose-1:6-diphosphate gave a perithecial count of 7.7 (approximately 0.005 % ester); while the calcium fraction (perithecial count 7.1) contained approximately 9.5 p.p.m. calcium.

On the basis of these results a medium was prepared to simulate the jute extract, i.e. containing 0.025 % glucose-6-phosphate, 0.005 % fructose-1:6-diphosphate (as potassium salts) and 9.5 p.p.m. calcium (as CaCl_2). (It is probably impossible to ensure complete absence of calcium from the materials used, and the calcium content of the medium must therefore be regarded as approximate.) Triplicate plates of this medium, triplicate plates of medium with jute extract, and plates of basal medium alone were tested in the usual manner and perithecial counts performed after 72 hr. On the jute extract medium the colonies reached a diameter of 40 mm. in 72 hr., while those on the reconstituted medium were somewhat smaller (35 mm.). Perithecial frequencies were almost the same (18.6 and 18.3 respectively), but the average size of the perithecia was considerably greater in the presence of jute extract. Thus it was concluded that as to speed of formation and numbers of perithecia the defined medium was equivalent to the jute extract. In two respects, however, a difference was noted, namely that mycelial growth was significantly greater and the perithecia themselves were much larger in presence of jute extract than of the mixture of sugar phosphates + calcium (cf. p. 303). It seemed likely that the jute extract contained some nutrient or stimulant favourable to the growth of all tissues but not concerned specifically with formation of perithecia. The effect of jute extract on vegetative growth has been noted before (Buston & Basu, 1948).

It appeared from the standard curves that the amount of each factor in the sample of jute extract used was not, except for calcium, very near its optimum concentration when acting alone. Tests were made with media in which were incorporated various combinations of the three substances in the estimated optimum quantities. In no instance did the production of perithecia equal that of a jute extract control, although the combination of all three was more effective than that of any pair. On the medium containing the two sugar phosphates (without calcium) perithecial frequency was 5.3; on that containing either phosphate + calcium, 6.3; on that containing all three factors, 7.3; and on the jute extract medium 12.1. (In these particular tests the perithecial frequency was unusually low even with jute extract. This was subsequently found to be due to the use of an inoculum from an older culture than usual. It was found desirable always to use an inoculum from a 7-day culture.)

DISCUSSION

It appears that calcium ion, glucose monophosphates and fructose diphosphate may all contribute to the chemical processes which underlie the formation of perithecia, and that this (though not necessarily the total growth) is most rapid and vigorous when all three substances are present in the medium. No explanation of this observation is offered, nor of the fact that a somewhat precise balance of the three is apparently beneficial. The effect of an aqueous extract of jute fibre, which happens to provide a very effective mixture, is reproduced as regards rapidity of formation and number of perithecia by a combination of the three substances mentioned. There is no evidence of the existence in jute extract of any specific 'fruiting factor'. Glucose-1-phosphate is absent from jute extract, but appears to be about as effective as the 6-phosphate which is present; the two are probably readily interconvertible by the organism, as noted by Hawker (1948) for *Melanospora destruens*. Although of several natural extracts examined that from jute was most effective, it is not suggested that it provides the maximum effect possible.

We wish to thank Dr W. G. Macmillan and Dr S. N. Basu of the Indian Jute Mills Association Research Institute, Calcutta, for a generous supply of jute fibre, and Prof. W. T. J. Morgan, F.R.S., for a gift of hexose phosphates.

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(Received 23 September 1952)

HOWARD HUGHES, W. (1953). *J. gen. Microbiol.* 8, 307-309.

The Origin of the L-form Variants in Anaerobic Cultures of *Bacterium coli*

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SUMMARY: When a strain of *Bacterium coli* derived from a single cell is grown under conditions approaching anaerobiosis, three different types of cell can be demonstrated: cells which divide to give the normal colonies; L-form; and an intermediate type which appears normal, divides once giving two daughter cells, one of which continues to divide normally while the other becomes an L-form.

A culture of a *Bacterium coli*, faecal type 1 ('River Brent'), was grown on a slide by the method of Fleming, Kramer, Voureka & Hughes (1950). Growth took place freely at room temperature, and individual organisms could be observed and photographed by phase contrast. It was noticed that a small number of the organisms failed to give normal colonies under these conditions of culture and instead grew out into so-called L-forms. See Pl. 1, fig. 1.

L-form is here used in the more recent and wider sense of the term. Originally the large form of *Streptobacillus moniliformis* alone carried this description and in that organism L colonies as well as abnormal cellular units occur. Recently the term has been used to define any organism which has a similar morphology whether or not colonies occur made up entirely of these forms. The essential feature of an organism which would qualify it for inclusion as an L variant would be that growth continued while division was inhibited. For a discussion of this phenomenon see Pulvertaft (1952).

It was at first assumed that these L-forms were contaminants. Single cells were therefore isolated, cultures grown from them for 3-5 hr. and the test repeated. The same appearances were noted in 3.75% of 1800 observed organisms. It appeared, therefore, that the original cell had given rise to other cells to which the environment was inimical in that division was inhibited in those organisms. Zelle & Lederberg (1951) noted the existence of lethal variants in their cultures under rather similar conditions but do not appear to have investigated them further.

The particular factor in the environment which inhibits division in these predisposed variants was investigated. There was no association between a tendency to form excessive numbers of L-forms on solid medium and penicillin sensitivity. The pH value of the medium was not responsible, nor had agar itself any action, as gelatin could be substituted for it. The degree of anaerobiosis, however, appeared to influence the result; on uncovered blocks of agar the L-forms have not been noticed. Broth cultures normally did not give L-forms with this strain but when iron strips were added then L-forms appeared. Broth cultures grown in anaerobic jars showed good L-forms

provided they were examined immediately after removal from anaerobic conditions on to slides. As soon as there was an adequate supply of oxygen available the L-forms resumed division and gave only small forms. The type of division noticed here was the same as with penicillin-induced L-forms in the presence of penicillinase (Fleming *et al.* 1950), namely, the large bacillus broke down rapidly into a number of daughter cells, suggesting that cell division had been inhibited in both cases at a late stage.

In making slide cultures Brewer's medium was substituted for nutrient agar with good results, and probably the medium of choice in demonstrating the phenomenon would consist of a similar formula with rather more agar in it.

The appearances seen with the various cultures of *Bact. coli* 'Brent' are in no way peculiar to that strain. Other coliform organisms, including *Bact. coli* strain K. 12, show similar changes under anaerobic conditions. These changes are not limited to artificial culture but can be seen in direct smears of pus.

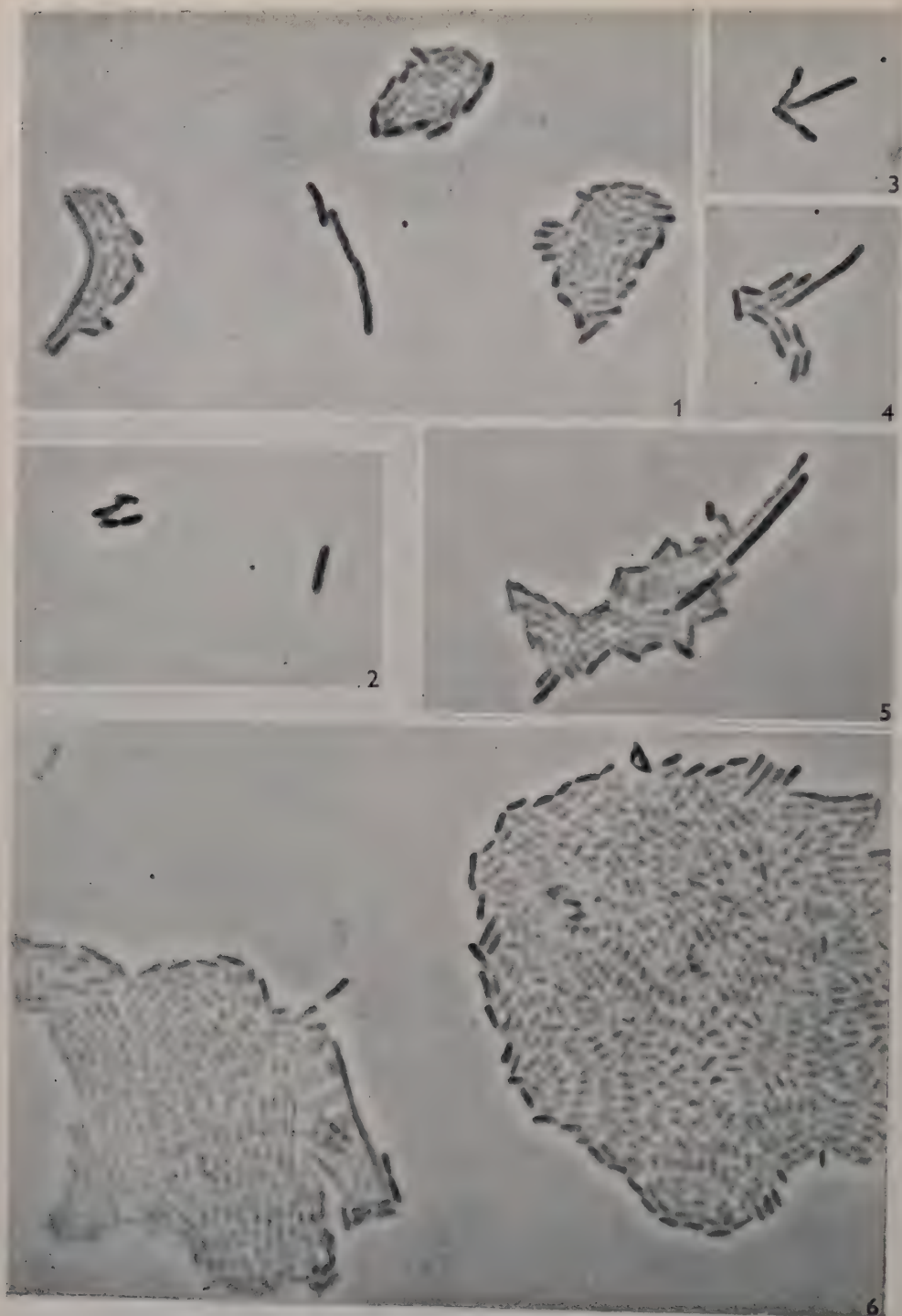
A second observation was made at the same time. The remaining apparently normal cells in the cultures divided, but nearly 17% (169 of 1000 colonies) were seen to have given rise at this stage to cells sensitive to the anaerobic conditions. The majority of cells divided into two daughter cells both of which redivided, but these aberrant cells gave two daughter cells, one of which divided normally and the other grew out without further division into an L-form. Once this L-form had been produced the normal cell associated with it divided to give a normal colony arranged alongside it, and no other L-forms appeared in that group until the culture had become confluent and further observation was not practicable. See Pl. 1, figs. 2-6.

The L-forms could be separated from the normal small forms by the use of the micromanipulator. On transfer to broth they reverted to normal, giving cultures which, in fluid media, were indistinguishable from those of the parent strain. However, on transfer to slide cultures again the difference in susceptibility to anaerobic conditions was immediately apparent. The comparison is summarized in Table 1.

Table 1. *Comparison of single cell parent-culture with subculture from a single L-form*

Strain		%		%
Brent I	Immediate L-forms	3.16	Latent L-forms	19.5
L-form, 4E	Immediate L-forms	14	Latent L-forms	49.6

Subcultivation, however, showed that regression in the selected strain was taking place for, after only four or five overnight cultures, the percentages were back to the level of the parent strain. The small forms selected from slide cultures in which the L-forms had already appeared might have been assumed to give a strain which would give no more L-forms, but this was not so. While the percentages of direct L-forms fell to a fraction of 1% and it was often difficult to find any, the latent L-forms appeared in all the cultures examined.



W. HOWARD HUGHES—L-FORM OF *BACT. COLI*. PLATE I

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EXPLANATION OF PLATE

Original photographs by phase contrast $\times 1200$. Not all are taken from the same field but are selected as typical.

- Fig. 1. The three colony types in strains grown from single cells of *Bact. coli* in anaerobic cultures. Left and below, normal colonies. Centre: two early L-forms developing from a pair of organisms. Right: a colony of one L-form and the progeny of one normal cell, both types arising from division of a single organism.
- Fig. 2. Failure of the first division of an incipient L-form. Normal first and second divisions in sister cells.
- Figs. 3-5. Development of the mixed colony from a single organism. Fig. 3. Stage of third division. Fig. 4. Stage of fifth division. Fig. 5. Stage of seventh division. The solitary L-form is now vacuolated.
- Fig. 6. Later colonies, that on the left still showing an L-form at the edge, that on the right being normal.

(Received 23 September 1952)

Lactobacillus parvus n.sp. Isolated from Beer

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SUMMARY: An organism isolated from a top-fermentation beer was found to be a new heterofermentative species of *Lactobacillus*. It appears as very small rods of length 1.0-1.5 μ . and width 0.8-1.0 μ . and forms chains. It ferments only glucose, maltose and sucrose and has been named *L. parvus*.

During investigations of lactic acid bacteria which occur as contaminants in breweries and which can cause sourness in beer, Walker & Parker (1943) isolated thirty-three organisms in pure culture. One of these, provisionally designated D4, has now been shown to have characteristics markedly different from those of other species of *Lactobacillus* previously described in the literature.

METHODS

The behaviour of the organism was observed in the following media: beer, hopped and unhopped, nutrient broth and yeast-extract peptone glucose broth (YEPG). Solid media were prepared by addition of agar or of gelatin to these liquid media. The organism usually does not grow in unhopped brewery wort but occasionally it has shown very feeble development in this medium and in this respect it is unlike many other lactic acid bacteria to be found as infections in yeast and in beer. This is not a pH effect because the samples of wort used had pH values lying between 4.9 and 5.4 and the organism grows strongly in beer at pH 4.9-5.4. Wort is probably deficient in a growth factor or factors required by this bacterium. The capacity of the bacterium to ferment carbohydrates was observed in a casein double digest prepared according to the directions of Davis (1939) and supplemented by addition of yeast autolysate (1 ml./100 ml. digest).

DESCRIPTION OF ORGANISM

Morphological characters

Shape, size and arrangement of cells: in unhopped beer after 24 hr. at 30° the majority of the cells were 0.8 \times 1.0 μ . with some up to 1.5 μ . in length and of width 0.9 μ . Single cells, and chains containing up to twenty cells, were noted. All cells were non-motile.

Staining: the organism was Gram-positive. Attempts to detect capsules, endospores and flagella failed.

Cultural characters

Observations of growth on solid media were made on cultures which had been incubated in atmospheres of CO₂ (85 vol.) plus air (15 vol.). Liquid media after inoculation were incubated in air. In all cases, with the exception of

growth in beer, which was observed at 30°, and of growth on YEPG gelatin, which was observed at 21°, the temperature of incubation was 25°.

Streak on beer agar after 4 days showed strong growth in the form of opaque, glistening, discrete colonies, of 1–2 mm. diameter. Streak on nutrient agar at 7 days showed slight nodose growth. Streak on YEPG agar at 7 days showed oval and circular discrete colonies of *c.* 1.5 mm. diameter and with yellow centres. Stab in YEPG agar after 5 days exhibited strong growth along the line of inoculation, without surface growth. Stab in YEPG gelatin after 5 days: growth was continuous to beaded along the line of inoculation and there was also slight surface growth.

Unhopped beer: at 3 days there was a fair amount of deposit giving rise, when agitated, to a uniform billowy turbidity. Nutrient broth permitted only a weak development of growth, seen at 7 days as a slight deposit, the liquid being clear. Growth in YEPG broth was moderately strong. A deposit separated on the side of the tube and when disturbed set up a non-silky turbidity.

Physiological characters

Relation to temperature: in unhopped beer the optimum range for growth was 25–34° with little or no growth below 14° or above 37°. Relation to oxygen: facultative anaerobe. Relation to pH value: in unhopped beer optimum pH value is 5.3; growth does not occur below pH 4.3 or above pH 6.9. Resistance to heat: killed by heating for 15 min. in unhopped beer at 60–65°. Resistance to hop antiseptic: growth markedly restricted by low concentrations (*c.* 1:10,000, w/v) of humulone (the principal bacteriostatic constituent of the hop).

Biochemical characters

Catalase reaction: negative. Acetylmethylcarbinol not formed. Nitrate not reduced to nitrite. Indole not formed. Gelatin not liquefied. Litmus milk not changed.

Carbohydrates utilized. On casein double digest + yeast autolysate, the organism attacked glucose, maltose and sucrose with production of acid, but gas was not detected by Durham tubes. On a xylose medium there was slight growth but acid was not formed. Arabinose, rhamnose, fructose, mannose, galactose, lactose, mannitol, dulcitol, sorbitol, inositol, salicin, inulin, trehalose, raffinose and dextrin were not attacked.

Nature of the acid produced from glucose. The lactic acid formed was converted to acetaldehyde and was estimated as such, after deproteinization of the medium. Another sample of medium was deproteinized, acetic acid separated by distillation in the presence of sulphuric acid and estimated volumetrically. The molar ratio of lactic acid: acetic acid was found to be 100:48. In a later experiment lactic acid was isolated as the zinc salt according to the procedure of Pederson, Peterson & Fred (1926); the free acid was optically inactive.

CLASSIFICATION

Since the organism was rod-shaped, non-motile, non-sporing, Gram-positive, facultatively anaerobic and catalase-negative with ability to ferment carbohydrates but not to reduce nitrate to nitrite, it was placed in tribe Lactobacillae Winslow *et al.* of the family Lactobacteriaceae Orla-Jensen. The production of large quantities of lactic acid by the organism further placed it in the genus *Lactobacillus* Beijerinck.

The organism was compared with species of *Lactobacillus* described in the literature (Pederson, 1938; Orla-Jensen, 1942; *Bergey's Manual*, 1948; Shimwell, 1949) and *L. brevis* was found to be the organism which it most resembles. However, the following major differences exist: *L. brevis* is seen as rods 2–4 μ . in length, whereas D4 is seen usually as very short rods about 1 μ . long, with some rods up to 1.5 μ . in length. In arrangement *L. brevis* occurs as single cells and some small chains, D4 forms definite chains of as many as twenty cells. In gelatin stab the growth of *L. brevis* is filiform while that of D4 is continuous and beaded. *L. brevis* produces acid in milk and attacks arabinose, xylose, glucose, fructose, galactose and maltose. Usually the attack of *L. brevis* on arabinose is vigorous and occurs with production of gas. D4 does not produce any change in milk and attacks only glucose, maltose and sucrose, without gas production. We think that these differences are sufficiently large to allow of species differentiation. Accordingly, we propose D4 as a new species, under the designation *L. parvus*, from the very small size of the cells.

***Lactobacillus parvus* n.sp.**

Rods, 1.0–1.5 μ . in length, width 0.8–0.9 μ ., occurring singly and as chains of as many as twenty rods. Non-motile. Capsules, endospores and flagella not detected. Gram-positive. Colonies on beer agar, nodose, opaque, glistening, 1–2 mm. diameter. Facultative anaerobe. Optimum temperature 25–34°, minimum 14°, maximum 88°. Optimum hydrogen-ion concentration 5.3.

Grows well in unhopped beer and in yeast-extract peptone glucose broth. Growth often appears as a deposit on the side of the tube. Does not grow in most samples of brewers' unhopped wort (a malt-extract solution) or in milk. Attacks glucose, maltose and sucrose with production of acid but not of gas. Has no action on arabinose, rhamnose, fructose, mannose, galactose, lactose, mannitol, dulcitol, sorbitol, inositol, salicin, inulin, trehalose, raffinose and dextrin.

Heterofermentative on glucose media, producing optically inactive lactic acid and acetic acid. Isolated at Manchester from sour beer, March 1942. Subcultures from the type culture have been deposited at the National Collection of Industrial Bacteria, The Chemical Laboratory, Department of Scientific and Industrial Research, Teddington, Middlesex (where its number is NCIB 8516), at the National Institute for Research in Dairying, Shinfield, Reading and at the Laboratories of the Brewing Industry Research Foundation, Nutfield, Surrey. Subcultures have been sent from the National Collection of Industrial Bacteria to the American Collection of Type Cultures.

The authors desire to thank Dr J. Tosic for assistance in the identification of the new organism.

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(Received 2 October 1952)

The Influence of pH Value and Aeration on the Growth of *Aerobacter aerogenes* and *Bacterium coli* in defined Media

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SUMMARY: The total amount of growth of *Aerobacter aerogenes* and *Bacterium coli* in glucose ammonium salt media which provided substrates in excess was dependent on the initial pH value in aerated and unaerated cultures. Total crops in aerated cultures were always higher for any particular initial pH value, and for cultures inoculated at pH values less than 7.0 the pH fell to lower values in aerated than in unaerated media. Growth of a culture of *A. aerogenes* in its (unaerated) stationary phase resumed on aeration; when the pH value at inoculation was 5.0-6.0, the pH continued to fall; when the pH value at inoculation was 6.1-7.0, the pH rose slightly. Growth of similar cultures of *Bact. coli* lagged when aerated; pyruvate concentrations immediately increased and pH values fell when glucose was in excess but not when it was limiting for growth. The concentration of formic acid produced by fully grown cultures was sufficient to abolish growth in fresh media below pH 5.25 but not at pH 7.0. These observations are discussed in relation to views on the influence of aeration on growth.

Relationships between total growth and initial pH value of citrate media were established for aerated and unaerated cultures of *A. aerogenes*. Growth in unaerated media ceased at pH values slightly higher than those at inoculation, leaving some citrate unconsumed. Aeration then caused growth resumption accompanied by consumption of citrate and a further increase in alkalinity.

Although the influence of pH value on bacterial growth has been frequently observed, Stephenson (1949) stated that much published work is vitiated because it takes into account only the initial pH value. Gale & Epps (1942) followed pH changes during growth of *Bacterium coli* in complex media. Lodge & Hinshelwood (1939) found that stationary populations of *Aerobacter aerogenes* in aerated glucose ammonium salt medium were profoundly influenced by the initial pH value of the medium. We have measured pH changes during growth in various defined media with and without aeration. Ravin (1952) suggested that growth cessation in unaerated media is due solely to development of adverse pH values. The increased bacterial crop on aeration may be due to a more favourable pH value which is achieved when accumulated organic acids are consumed. We have tested this speculation by measuring pH changes when unaerated cultures in their stationary phase were aerated.

METHODS

The organisms used throughout this work were *A. aerogenes* NCTC418 and *Bact. coli* NCTC5928. The media used contained KH_2PO_4 , 5.4 g.; $(\text{NH}_4)_2\text{SO}_4$, 1.2 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g., made to 1 l. with glass-distilled water and, unless otherwise stated, the required carbon sources were added to give concentrations of 12 g. glucose and 35.3 g. Na citrate dihydrate/l. The pH value was adjusted to that required by the addition of 5N-NaOH or 5N-HCl. Cultivation was at 37° in either 6 × 1 in. or 8 × 1½ in. Pyrex tubes. Cultures were aerated by passage of a gentle stream of sterile air. Methods of obtaining growth curves and precautions necessary to ensure reproducibility have already been described (Dagley, Dawes & Morrison, 1950*a*). Samples for pH and other determinations were obtained by centrifugation of cultures. Marconi type TF889 and Muirhead type D417A pH meters with glass electrodes were used for pH measurement. Titration curves for the media were prepared against 5N-NaOH and 5N-HCl to enable pH changes to be expressed as amount of acid or alkali produced per cell.

Pyruvic acid was determined by the toluene extraction method of Friedemann & Haugen (1943) and citric acid by the method of Perlman, Lardy & Johnson (1944). Glucose concentrations were determined by the Somogyi (1937) method and ammonia by nesslerization. Formic acid was determined by the A.O.A.C. (1945) method and the acetic acid by the difference between the total volatile acid concentration, obtained by steam distillation and titration with 0.1N-NaOH, and the formic acid concentration. Acetoin was determined by the method of Happold & Spencer (1952*a*).

RESULTS

Relationship between total growth and initial pH of glucose medium

Growth of cultures containing excess substrates (Dagley, Dawes & Morrison, 1951) at a series of initial pH values was followed turbidimetrically until growth ceased. In agreement with the results of Lodge & Hinshelwood (1939) we found that the crop was considerably affected but that the mean generation time was not. Final bacterial populations for aerated and unaerated cultures are shown in Fig. 1. For both organisms over the whole pH range higher final populations were obtained in aerated than in unaerated media. When the initial pH value was <6, *A. aerogenes* grew to higher populations than *Bact. coli* in both aerated and unaerated cultures. From the titration curve for the medium and measurements of pH values at the cessation of growth, the amount of acid formed was calculated. The amount of acid accumulated per million cells is plotted in Fig. 1; for *A. aerogenes* this value falls sharply to a minimum at pH 5.8. Determinations of acetoin in growing cultures showed a rapid increase in concentration when the pH fell below 6.0; the pH optimum for the reaction is given as 5.6–6.0 (Silverman & Werkman, 1941; Happold & Spencer, 1952*b*; Juni, 1952) and this minimum in the curve of acid production for *A. aerogenes* may probably be attributed

to acetoin formation. For both organisms acid production in unaerated conditions was higher and in all cases maxima were given at *c.* pH 7.5.

Effect of aeration on pH value of glucose medium

Measurements of pH value within 1 hr. of growth cessation (Table 1) showed that with initial pH values <7 aerated cultures continued to grow when the pH had fallen to a value which inhibited growth in unaerated media. From these results it appeared that growth with aeration was less sensitive to high acidity than semi-anaerobic growth. We therefore investigated changes

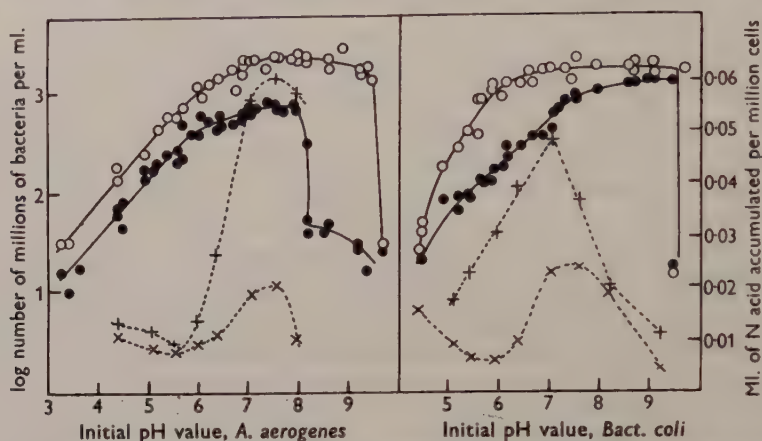


Fig. 1. Relationship between initial pH of glucose ammonium salt medium, total growth and acid accumulation, for *A. aerogenes* and *Bact. coli* in aerated and unaerated cultures. Growth: aerated, ○; unaerated, ●. Acid accumulation, culture: aerated, +; unaerated, ×.

in pH value and bacterial population when cultures which had ceased to grow in unaerated conditions were aerated. When the initial pH value of an *A. aerogenes* culture was <6.1, the pH value continued to fall on aeration, whilst a rise in pH value occurred when the initial pH value was between 6.1 and 7.0. For this organism an increase in cell population occurred on aeration at all pH values investigated. Representative results for three pH values are shown in Fig. 2.

Table 1. *Initial and final pH values of Bact. coli and A. aerogenes cultures grown with and without aeration in glucose ammonium salt media*

Initial	pH values						
	5.1	5.5	6.0	6.4	7.1	7.6	8.2
<i>Bact. coli</i>							
Final (not aerated)	4.4	4.6	4.7	5.3	6.0	6.5	7.0
Final (aerated)	4.1	4.1	4.6	4.9	5.8	6.3	6.7
<i>A. aerogenes</i>							
Final (not aerated)	4.1	4.4	5.0	5.4	5.7	5.9	6.8
Final (aerated)	3.8	3.8	3.8	4.7	5.7	6.0	6.9

The initial pH value profoundly influences the balance of end products in bacterial fermentations (Tikka, 1935). We considered it probable that when the pH value increased during aeration, organic acids were being consumed in growth at a rate more than sufficient to counterbalance production of acid by resumed glycolysis, and that in those cases where the pH value fell, glycolysis exceeded acid consumption. In Fig. 2 it is seen that growth did

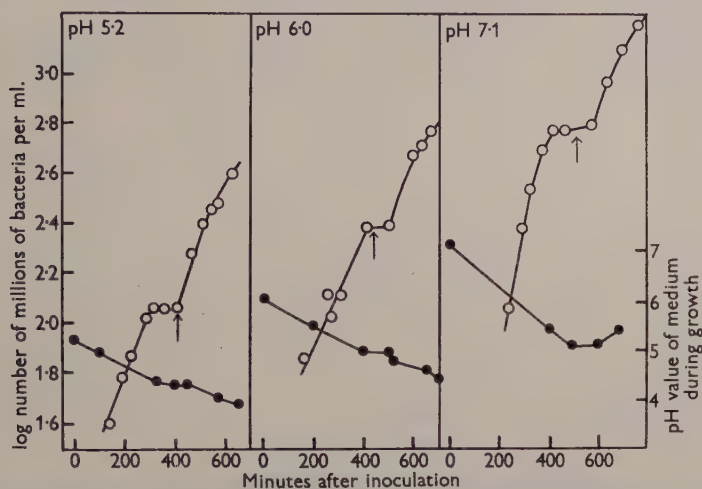


Fig. 2. Changes in pH during growth of *A. aerogenes* in glucose ammonium salt medium, at three different initial pH values, to the unaerated stationary phase followed by aeration and further growth. Growth, ○; pH, ●. Arrows indicate start of aeration.

not resume immediately on aeration; Lwoff & Monod (1947) reported prolonged lag periods for *Bact. coli* in aerated media. Such delays in growth of our strain of *Bact. coli* were usually of longer duration than those for *A. aerogenes* and permitted us to study the resumption of glycolysis in the absence of growth. The relation between stationary populations and glucose concentrations in the present medium has been reported previously (Dagley *et al.* 1951; Ravin, 1952) and enabled us to measure pyruvate concentrations and pH values for cultures of *Bact. coli* containing amounts of glucose less than, and in excess of, full growth requirements (Fig. 3). In no case was there an increase in pH value on aeration, and pyruvate concentration increased immediately in those cultures where excess glucose remained, but not in those from which glucose was exhausted.

It is seen in Table 1 that growth may cease at a pH value which permits growth to proceed in a fresh medium; e.g. an unaerated *A. aerogenes*, initially at pH 7.2, ceased to grow at pH 5.8, whilst a culture initially at pH 5.8 grew until the pH fell below 5. This suggests that products of metabolism became increasingly toxic as the pH value decreased. Both these organisms produce formic acid, and Gale & Epps (1942) showed that 0.2% (w/v) sodium formate stopped growth of *Bact. coli* below pH 6.2. Determinations of formic acid in filtrates from fully-grown cultures of *A. aerogenes* and *Bact. coli* showed the

presence of 470 and 530 mg. formic acid/l., respectively. The effect of these concentrations of formic acid on growth of these organisms in the range pH 5.0–6.0 is shown in Table 2. Formic acid is increasingly toxic as the pH value decreases, and growth is completely inhibited at pH 5.25 and 5.0 for *Bact. coli* and *A. aerogenes* respectively.

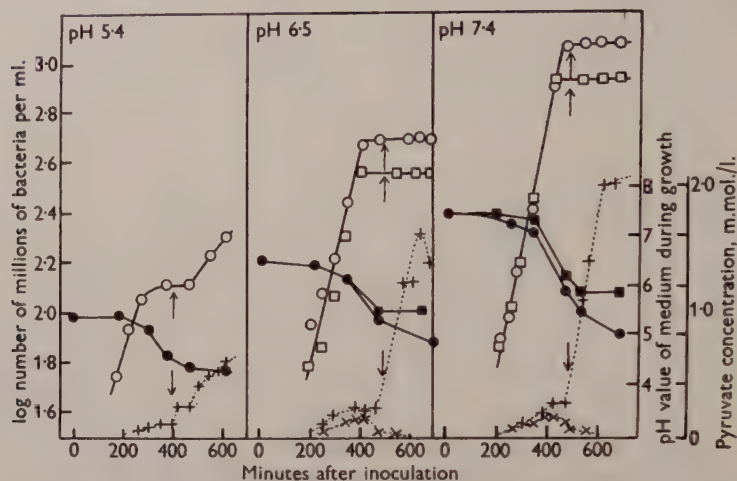


Fig. 3. Changes in pH and pyruvic acid concentration during un-aerated growth and subsequent aeration of stationary phase cultures of *Bact. coli* in glucose ammonium salt media at three different initial pH values. Glucose limiting growth: growth, □; pH, ■; pyruvate concentration, x. Glucose in excess of growth requirements: growth, ○; pH, ●; pyruvate concentration, +. Arrows indicate start of aeration.

Table 2. Effect of formic acid concentrations found in fully grown cultures of *Bact. coli* and *A. aerogenes* in un-aerated glucose ammonium salt medium on the growth in similar fresh media over the pH range 5.0–6.0

Initial pH value	<i>Bact. coli</i>		<i>A. aerogenes</i>	
	Formate (530 mg./l.) present	No formate	Formate (470 mg./l.) present	No formate
	Stationary populations of bacteria in millions/ml.			
5.0	0	50	0	160
5.25	0	80	75	200
5.50	20	100	350	310
5.75	90	180	460	370
6.0	160	200	490	410

Relation between total growth of Aerobacter aerogenes and initial pH value of citrate medium

Citric acid as source of carbon for growth was studied for two reasons:

- (i) the pH value increases during growth on citrate in contrast to glucose,
- (ii) *A. aerogenes* grows well both aerobically and anaerobically in this medium.

Final bacterial populations were measured in unaerated media at various concentrations of citrate and initial pH value of 7.0. They increased linearly with concentration up to 6×10^{-2} M-citrate, at which concentration a population of 950×10^6 bacteria/ml. was supported. Higher concentrations of citrate produced no increase in crop. With aerated media at initial pH value 7.0 the linear relationship ceased at 3.6×10^{-2} M-citrate, with a population of 1220×10^6 bacteria/ml. For *A. aerogenes* the following comparison may be made of the substrate concentrations required to support a population of 10^6 bacteria/ml.: with aeration, glucose 0.66×10^{-5} M citric acid 2.80×10^{-5} M; without aeration, glucose 1.66×10^{-5} M citric acid 6.95×10^{-5} M.

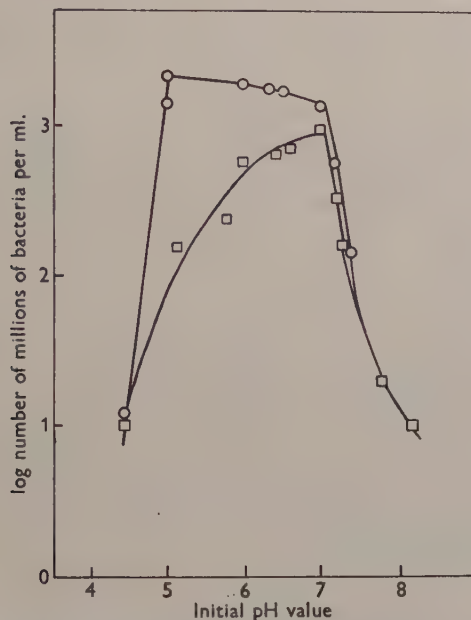


Fig. 4. Relationship between initial pH of citrate-ammonium salt medium and total growth of *A. aerogenes* in unaerated and aerated media. Growth: aerated, ○; unaerated, □.

Measurements of final populations in aerated and unaerated media containing citrate in excess of growth requirements (35.3 g./l.) were made over a range of initial pH values (Fig. 4). For both aerated and unaerated citrate media there was a sharp decrease in the stationary population when the initial pH values were between 7.0 and 7.5. With initial pH values between 5.0 and 7.0 there was a contrast between unaerated and aerated cultures; for the unaerated cultures there was a decrease of population with decrease of initial pH value and for the aerated cultures an increase. Thus at low initial pH values the increase in crop due to aeration was even greater for citrate than for glucose. Table 3 records pH measurements for cultures in their stationary phase after growth with and without aeration for different initial pH values. In all cases there was an increase in pH value which was

most pronounced in aerated cultures. Changes in pH value of unaerated cultures were so small that cessation of growth could not be attributed solely to this factor. When unaerated cultures at initial pH value 6.85 were aerated in their stationary phase growth resumed and the pH value increased (Table 4). Simultaneous determinations of citrate indicated that citrate decomposition was also resumed on aeration.

Table 3. *Initial and final pH values of A. aerogenes cultures grown with and without aeration in citrate ammonium salt media*

	pH values				
Initial	5.1	5.8	6.3	6.6	7.0
Final (not aerated)	5.3	5.9	6.5	6.8	7.0
Final (aerated)	5.3	6.5	7.6	8.2	7.8

Table 4. *Changes in pH value and citrate concentration when A. aerogenes cultures are grown without aeration to their stationary phase, followed by aeration with resultant growth resumption to their aerated stationary phase*

Time of determination of pH value	pH value		Citrate concentration (mg./ml.)	
	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>
At inoculation	6.85	6.85	15.30	15.30
At stationary phase not aerated	7.75	7.60	5.42	5.20
At stationary phase after aeration	8.90	8.80	3.14	2.86

a = 25 ml. culture; *b* = 100 ml. culture.

Table 5. *Effect of acetic acid concentration found in stationary phase unaerated citrate ammonium salt cultures of A. aerogenes on the growth in similar fresh media at various pH values*

	Initial pH				
	6.2	6.4	6.6	6.8	7.1
Stationary populations of bacteria in millions/ml.					
Acetic acid (3.46 g./l.) present	5	13	128	380	600
Acetic acid absent	545	575	725	910	910

Since changes in pH value alone did not account for growth cessation in unaerated media containing excess citrate we investigated the possibility of the formation of toxic products. In the mechanisms proposed by Deffner (1938) and by Brewer & Werkman (1939) citrate is split initially, in anaerobic conditions, to oxaloacetic and acetic acids. Although the oxaloacetic acid inhibits reactions of importance in bacterial metabolism (Stone & Wilson, 1952; Pardee & Potter, 1948) additions of oxaloacetate up to 80 mg./l. increased both rate of growth and final population. The ability of oxaloacetate to promote growth on addition to defined media has been reported previously (Ajl & Werkman, 1948; Dagley, Dawes & Morrison, 1950*b*). Determinations of acetic and formic acids in fully grown unaerated cultures (initial pH 7.0)

gave values of 3.46 and 0.91 g./l., respectively. The addition of this concentration of acetic acid to unaerated cultures inhibited growth in the pH range 6.2-7.1; the effect was more pronounced at low pH values (Table 5).

DISCUSSION

Cessation of growth of *Bact. coli* and *A. aerogenes* in glucose ammonium salt media when nutrients remain unconsumed was attributed to failure of hydrogen transfer from reduced diphosphopyridine nucleotide (DPN) to metabolites (Dagley *et al.* 1951). Production of toxic products was not considered responsible for the end of growth since sterile filtrates from such cultures promote growth (Dagley *et al.* 1950*b*). Such filtrates, however, were tested at pH values which were optimal for growth. The present work shows that formic acid, the toxicity of which increases rapidly as the pH value decreases, is produced in quantities sufficient to inhibit growth at the pH values reached in unaerated media. Inhibition of growth by other end products of metabolism may also increase as acidity develops, and an explanation is afforded of the observation that cultures may cease to grow at pH values which permit growth in fresh media. Ravin (1952) showed that increase of buffer capacity led to increased stationary populations in unaerated cultures and attributed stoppage of growth to the development of adverse pH values, and the resumption of growth on aeration to consumption of accumulated organic acids. We showed, however, that for certain initial pH values acidity continued to develop although growth resumed on aeration. Further, in the present and previous work (Dagley *et al.* 1951), estimations of pyruvate indicated that glycolysis was resumed on aeration, and it seems unlikely that this is without significance for growth.

The effect of acidity on the growth of *A. aerogenes* and *Bact. coli* was more pronounced with unaerated than with aerated cultures. Perhaps hydrogen transfer from reduced DPN to metabolites is inhibited at pH values which permit hydrogen transfer to oxygen. Fowler (1951) showed, however, that anaerobic utilization of glucose by *Bact. coli* appears to be effected by an adaptive enzyme which does not function under aerobic conditions; inhibition by toxic products at low pH values may therefore occur at this point.

The factors which cause growth cessation in anaerobic citrate cultures cannot at present be completely evaluated. Although conditions inside the cell may differ greatly from those outside, measurement of changes of pH value in the external fluid do not support the view that the development of adverse pH values causes growth to cease and the resumption of growth on aeration certainly cannot be attributed to consumption of organic acids formed as fermentation products. Although we showed that acetic acid was produced anaerobically in amounts sufficient to cause inhibition, growth was not abolished at pH 7.

We wish to express our gratitude to the Carnegie Trust for the Universities of Scotland for a personal grant to one of us (E.A.D.) towards the cost of materials for this work.

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(Received 3 October 1952)

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The Antimycobacterial Activity of Tissue Extracts and Surface-active Agents in Dubos's Medium

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SUMMARY: Purified tissue enzyme preparations, particularly bovine pancreatic deoxyribonuclease, have been shown to inhibit specifically pathogenic strains of *Mycobacterium tuberculosis*. Destruction of the enzymic activity did not influence this effect. Inhibition in Dubos's medium was markedly increased by Tween 80 but not by other surface active agents. Antibiotic potentiation by Tween 80 alone or combined with the tissue extracts was observed. The antimycobacterial activity of spermine was confirmed, but there was no evidence that it was present in our extracts and the inhibitory substance has not yet been identified.

Pirie (1935) reported that extracts of pig pancreas removed the pathogenicity of vaccinia and Rous tumour viruses. She suggested that the inactivation of some viruses by trypsin might be due to the action of fatty acids and lecithin, since crystalline trypsin prepared by Northrop (1932) had no effect on these viruses. Day & Gibbs (1930) have described a canine pancreatic secretion obtained directly from the pancreatic duct, which inhibited tubercle bacilli (strain H37Rv) but not staphylococci. More recently, Hirsch & Dubos (1952) extracted spermine from beef kidney; this polyamine, which is widely distributed in animal tissues, was found to possess a remarkable specific inhibitory activity against pathogenic mycobacteria.

Soltys (1952) has reported the isolation of a substance from bovine tuberculous lymph nodes which inhibited bovine and human tubercle bacilli *in vitro* and *in vivo*. Although this factor was not identified, he suggested that it might be either a form of immune body or a substance similar to spermine.

During preliminary experiments with the medium of Dubos & Davis (1946) containing 0.05 % (w/v) Tween 80 (polyoxyethylene sorbitan monoleate) and inoculated with *Mycobacterium tuberculosis* H37Rv (NCTC7416), we observed that the addition of bovine testicular hyaluronidase or bovine pancreatic deoxyribonuclease completely inhibited growth; on replacing the surface active agent by 2 % glycerol, a similar concentration of these enzymes possessed no inhibitory activity. Further investigations were therefore undertaken in order to elucidate this interesting observation.

MATERIALS AND METHODS

Preparation of tissue extracts

The enzymes investigated for antibacterial activity were bovine pancreatic deoxyribonuclease, hog pancreatic lipase and bovine testicular hyaluronidase. The deoxyribonuclease was prepared from fresh bovine pancreas by extrac-

tion with 0.2 N-sulphuric acid and purified by fractionation with ammonium sulphate by the method described by McCarty (1946). The hyaluronidase was prepared from frozen bovine testes by extraction with 0.007 % (w/v) glacial acetic acid and purified by fractionation with ammonium sulphate by the technique adopted by Hahn (1943). The lipase was prepared from fresh hog pancreas which had been extracted with excess N-butanol and the residue vacuum dried.

Sterile preparations were obtained by filtering aqueous solutions of the freeze-dried enzymes through Ford 'S.B.' sterilizing pads.

Medium

Basal medium was essentially that used by Dubos & Davis (1946) and consisted of (w/v): casein hydrolysate (Benger's Ltd, Holmes Chapel) 0.1 %; $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$, 0.63 %; KH_2PO_4 , 0.1 %; $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.06 %; sodium citrate, 0.15 % in distilled water at pH 7.0. This medium was supplemented with 0.2 % (w/v) bovine albumin and either various surface active agents or 2 % (w/v) glycerol.

Albumin solution was prepared by adjusting the pH of a 5 % (w/v) solution of bovine albumin Fraction V (Armour Laboratories Ltd., London) in 2 % (w/v) sodium chloride to 7.0, warming the solution at 56° for 30 min. and sterilizing by filtration through a Ford 'S.B.' pad.

Surface active agents

(1) *Tween 80 solution*: the Tween 80 (polyoxyethylene sorbitan monoleate) used throughout the investigation was the purified product (G. T. Gurr Ltd., London), prepared specifically for use in media for the tubercle bacillus. Fresh 10 % (w/v) aqueous solutions of Tween 80 were prepared and autoclaved at 15 lb./sq.in. for 15 min. immediately before use.

(2) *Crill 20*: polyoxyethylene sorbitan monostearate (Croda Ltd., Goole).

(3) *Carbowax*: polyethylene glycol of molecular weight 600 (General Metallurgical and Chemical Co. Ltd., London).

(4) *Triton A20*: arylalkyl polyethoxyethanol (Rohm and Haas Co., Philadelphia).

The basal medium, in volumes of 5 ml., with or without glycerol, was distributed into 1 oz. McCartney bottles with metal screw caps and autoclaved at 15 lb./sq.in. for 15 min.; thereafter, the supplements of albumin and Tween 80 or other surface active agents were added aseptically.

Culture and inoculum

M. tuberculosis strain H37Rv (NCTC7416) was used; maintained on Petragnani (egg, potato-flour, skimmed milk) slopes. At monthly intervals the organism was subcultured into the liquid medium containing 0.05 % (w/v) Tween 80. The inoculum was well shaken with glass beads, allowed to stand for 1 hr. and then 0.1 ml. of the supernatant inoculated into 5 ml. of Dubos's medium. After 7-10 days incubation at 37° a dispersed growth was obtained.

The Breed count of this culture was adjusted so that 0.1 ml. delivered into 5 ml. of medium by means of a preset McClintock syringe (C. J. Hewlett and Co. Ltd., London) gave an inoculum of 5×10^5 to 1×10^6 organisms/ml.

Chromatographic method

The paper partition chromatographic technique described by Consden, Gordon & Martin (1944) was employed to give a one-dimensional chromatogram on Whatman no. 54 paper, using the upper layer of *n*-butanol + acetic acid + water mixture (4 : 1 : 5) as the mobile solvent. The paper was sprayed with ninhydrin (0.1 %, w/v, in butanol) and a comparison made with a sample of spermine phosphate (L. Light and Co. Ltd., Colnbrook, Bucks).

RESULTS

Inhibition of Mycobacterium tuberculosis in Dubos's medium by tissue extracts and surface active agents

In Dubos's medium containing 0.05 % (w/v) Tween 80, it was observed that the presence of bovine testicular hyaluronidase, bovine pancreatic deoxyribonuclease or hog pancreatic lipase, at a concentration of 3 mg. of enzyme in 5 ml. medium, completely inhibited the growth of *M. tuberculosis* (strain H37Rv). On replacing the Tween 80 by other surface active agents, i.e. 0.01 % (w/v) Crill 20, 0.05 % (w/v) Carbowax, 0.1 % (w/v) bile salts, 0.05 % (w/v) Triton A20, or by 2 % (w/v) glycerol, no inhibition of growth occurred in the presence of the enzymes. It was further demonstrated that Tween 80 and Triton A20 at a tenfold increase in concentration (0.5 %, w/v) possessed no inhibitory activity in the absence of the tissue extracts.

Inhibition of H37Rv was subsequently observed, even in the absence of Tween 80 and the other detergents, when a sixfold increase in concentration of pancreatic deoxyribonuclease and lipase was used.

Similar observations were made when Dubos's medium was supplemented with 10 % (w/v) horse serum.

The potentiation of the activity of penicillin against H37Rv by 0.05 % (w/v) Tween 80 reported by Kirby & Dubos (1947) was confirmed, but the surface active agent Triton A20 failed to enhance the inhibitory activity of either penicillin or the enzymes. It was observed, in addition, that pancreatic deoxyribonuclease, in the presence or absence of Tween 80, was capable of increasing the antimycobacterial activity of both penicillin and streptomycin, but similar synergistic activity could not be demonstrated with all the preparations of deoxyribonuclease examined. These results are shown in Table 1.

Enzymic activity and inhibitory effect

It was observed that several preparations of pancreatic deoxyribonuclease, although exhibiting similar enzymic activity when assayed by the reduction in viscosity of a deoxyribonucleic acid substrate (McCarty, 1946), possessed varying antimycobacterial activity, which in most cases was considerably potentiated by Tween 80. However, one preparation (batch C) produced no

inhibition when present in a concentration of 25 mg./5 ml., although it possessed an enzymic activity similar to the other preparations (batches A, B and D). This is shown in Table 2.

Table 1. *Potential of activity of streptomycin and penicillin against Mycobacterium tuberculosis H37 Rv by pancreatic deoxyribonuclease (batch D)*

	Minimal antibiotic concentration (units/ml.) to cause inhibition after 8 weeks incubation at 37°								
	Penicillin					Streptomycin			
	1000	100	10	1	0	1	0.5	0.25	0.125
(1) No Tween 80 or D-ase	+	+	+	+	+	-	+	+	+
(2) 0.025 % Tween 80. No D-ase	-	-	+	+	+	-	-	+	+
(3) 0.01 % Tween 80. No D-ase.	-	+	+	+	+	-	+	+	+
(4) 0.025 % Tween 80 + 3 mg./5 ml. D-ase	-	-	-	-	-	-	-	-	-
(5) 0.01 % Tween 80 + 3 mg./5 ml. D-ase	-	-	-	+	+	-	-	-	-
(6) No Tween 80 + 3 mg./5 ml. D-ase	-	-	+	+	+	-	+	+	+
(7) 0.05 % Triton A20. No D-ase	+	+	+	+	+	-	+	+	+
(8) 0.05 % Triton A20 + 3 mg. D-ase/5 ml.	-	+	+	+	+	-	+	+	+

Full growth = + ; no growth = -.

Table 2. *Minimal concentration of three preparations (A, B, D) required for complete inhibition of M. tuberculosis (H37 Rv) with and without the addition of 0.05 % Tween 80 after 3 weeks' incubation at 37°*

Culture medium	Mg./5 ml. broth		
	A	B	D
Dubos's (Tween 80 absent)	25	20	18
Dubos's + 0.05 % (w/v) Tween 80	6	3	3

Growth inhibition was later shown to be independent of enzymic activity since a similar effect occurred after the enzymes had been destroyed by heating at 56° for 30 min. on two successive days.

Specificity of the active inhibitory substance

The tissue extracts were found to possess activity against a virulent bovine strain of *M. tuberculosis* similar to that described for H37Rv. However, using high concentrations (18 mg./5 ml.) of the freeze-dried extracts in the presence or absence of 0.05 % (w/v) Tween 80, no inhibition could be demonstrated against the saprophytic *M. phlei* (NCTC525) or against *Salmonella typhi* (NCTC785), *Streptococcus salivarius* (NCTC445), *Bacterium coli-commune* (NCTC86), *Pseudomonas aeruginosa* (NCTC7244), *Klebsiella pneumoniae* 41 (NCTC7242) and *Staphylococcus aureus* (NCTC6571).

Comparison of bovine deoxyribonuclease with spermine

The antimycobacterial activity of pancreatic deoxyribonuclease was compared with spermine phosphate, which can be isolated from various tissues, including the pancreas (Fearon, 1947). Hirsch & Dubos (1952) claimed that

a concentration of 0.125 mg. of spermine phosphate in 5 ml. of Dubos's medium inhibited the growth of pathogenic mycobacteria but had no effect on other pathogenic organisms, and this was confirmed. In contrast to the findings with our tissue extracts, the activity of spermine was observed to be unaffected by the addition of Tween 80 to Dubos's medium.

Chromatographic analysis of preparation B (see Table 2) of pancreatic deoxyribonuclease failed to reveal the presence of spermine, and fractionation of this material by methods employed by Hirsch & Dubos, with a view to isolating spermine, proved unsuccessful, since the various fractions obtained failed to correspond chromatographically to spermine.

DISCUSSION

Arising from the work of Dubos (1945) and Dubos & Davis (1946), addition of the synthetic non-ionic detergent, Tween 80, to defined culture media for virulent tubercle bacilli has been widely adopted. Tween 80 appears to exert a stimulatory effect on the growth of tubercle bacilli and produces a diffuse homogeneous growth instead of the usual granular floccules. This surface active agent probably accelerates the growth of the organism by assisting the absorption of nutrients through the cell wall; at the same time, it produces a conveniently dispersed suspension of the tubercle bacilli. In a similar manner, Tween 80 could permit a more intimate contact between an antibacterial agent and the surface of the cell wall, and even enhance intracellular penetration of the inhibitory substance.

Youmans & Youmans (1948) reported that the presence of Tween 80 in a defined medium increased the tuberculostatic activity of fifteen (including chloramphenicol) out of twenty compounds, decreased the activity of three compounds and had no effect on two compounds.

Potentialiation of the antibiotic activity of penicillin (Kirby & Dubos, 1947) and streptomycin (Fisher, 1948) by Tween 80 against *M. tuberculosis* H37Rv has been previously reported; Bliss & Warth (1950) observed a similar effect on the activity of polymyxin D against *Bact. coli*. In our investigations, it seemed possible that Tween 80 likewise influenced the antibacterial activity of the purified enzyme preparations. In view of the reported potentialiation of antibacterial agents by Tween 80 and our failure to obtain a similar effect with other surface active agents, it is of interest to suggest reasons for this specific property of Tween 80.

Triton A20 is a powerful non-ionic wetting agent which, at a concentration of 0.05 % (w/v) in Dubos's medium, ensures a rapid and dispersed growth of *M. tuberculosis*, but we observed no effect on the activity of various antimycobacterial agents.

A significant difference between Triton A20 and Tween 80 is that the former contains no fatty acid group. There are grounds for suggesting that the oleic acid group in the Tween 80 molecule may play a delicate role in the metabolism of the tubercle bacillus since esters of lauric and palmitic acid (Tween 20 and Tween 40) have been shown to be inhibitory (Dubos & Davis, 1946). In the present investigation, Crill 20, a stearate, was itself inhibitory

at 0.05 % (w/v), permitted growth at 0.01 % (w/v), but failed to potentiate the activity of tissue extracts at the latter concentration. Dubos & Middlebrook (1947) have reported that long chain fatty acids, and oleic acid in particular, exert a dual effect on *M. tuberculosis*. In most media, particularly in the absence of albumin, their soaps are markedly inhibitory, but when supplied to the medium in a non-toxic form, for example as a water dispersable ester such as Tween 80, they can act as nutrients for mycobacteria.

All strains of tubercle bacilli were found by Dubos (1946) to be capable of utilizing oleic acid, but human and bovine strains are so susceptible to oleic acid that it is often very difficult to choose amounts sufficient to stimulate growth appreciably without reaching the toxic concentration. In view of the delicate balance which exists in regard to the oleic acid requirements of the pathogenic mycobacteria, it would appear advisable to confine the use of Tween 80 to the preparation of a homogeneous inoculum, and perhaps serious consideration should be given to its replacement by Triton A 20.

Hirsch & Dubos (1952) isolated a specific antimycobacterial factor, spermine, from beef kidney, but this substance is widely distributed in animal tissues (Table 3) and is found in greatest amounts in the prostate gland.

Table 3. *Representative values of spermine in mg./100 g. fresh tissue*

(from Fearon, 1947)

Human prostate	180	Human liver	10
Human pancreas	16	Ox pancreas	25-30
Testicle, spleen, kidney 1-7			

We have not been able to demonstrate the presence of spermine in the pancreatic enzyme preparations used in this investigation, and it would appear that an active thermostable principle, other than spermine, exists in certain enzymic tissue extracts which is capable of a highly specific antimycobacterial action in the presence of serum proteins. It is recognized that pancreatic tuberculosis is a rare disease and it may be suggested that this is due to the presence in the pancreas of a single, or several specific, antimycobacterial substances.

We wish to express our thanks to the Directors of Bengers' Ltd. for permission to publish these results, and to Dr C. Giles of the City General Hospital, Newcastle-under-Lyme and Dr A. J. McCall of the North Staffordshire Royal Infirmary, Stoke-on-Trent, for their interest in this work.

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(Received 7 October 1952)

Lactobacillus frigidus n.sp. Isolated from Brewery Yeast

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SUMMARY: Details are given of a new species of the genus *Lactobacillus* isolated from a sample of brewery yeast. The cells appear as short thick rods, mainly in pairs and small clumps. This organism, which ferments arabinose, xylose, glucose, fructose, mannose and sucrose, has the low optimum temperature of 22-23° and fails to grow above 30°. It has been named *Lactobacillus frigidus*.

During studies of lactic acid bacteria which occur as contaminants of brewery yeasts fifty-four cultures of the former were isolated (Bhandari, 1951); four of these consisted of rod-shaped organisms very similar to each other but unlike previously described lactobacilli. These cultures were provisionally designated HH1, HH2, HH3 and HH5, the last being chosen as the type. The characters and behaviour of HH5 are now described.

METHODS

The attributes of the organism were studied in liquid and in solid media made from beer (hopped and also unhopped) and from unhopped wort. Glucose nutrient broth was also used. The fermentation characteristics were observed in a medium which consisted of casein double-digest prepared according to Davis (1939), with the addition of yeast autolysate (1 ml./100 ml. digest).

DESCRIPTION OF THE ORGANISM

Morphological characters

Shape, size and arrangement of cells. In unhopped beer at 25° cells were seen as short rods, some being almost coccoid forms; their size was usually $0.7 \times 1.1 \mu$. Single cells, pairs, small clumps and chains of four to six cells were observed. The organism was not motile.

Staining. The cells were Gram-positive. Capsules, flagella and endospores were sought in cultures on the several suitable media but were not detected.

Cultural characters

Single colonies on solid media in an atmosphere of CO₂. After 7 days on beer gelatin plates at room temperature colonies were from 1 to 1.5 mm. in diameter, entire and colourless. Growth on beer agar slopes was weak.

Stab. Moderately strong beaded growth in beer gelatin, scanty near the surface.

Growth in liquid media at 25°. In 2-3 days the organism developed well in unhopped beer giving rise to the silky turbidity which characterizes the growth of several species of *Lactobacillus* in this medium; the liquid subse-

quently cleared when a small deposit formed. No growth occurred in hopped beer containing 0.003 % of humulone, which is the principal bacteriostatic constituent of the hop and exercises inhibitory effects on the growth of *Lactobacillus* species. HH 5 developed well during incubation for 3 days in unhopped wort, and growth was also strong in glucose Lemco peptone broth.

Physiological characters

Relation to temperature. In unhopped beer the optimum temperature for growth is 22–23°; minimum at 15°, maximum at 30°. *Relation to pH value.* In unhopped beer the optimum pH range is from 5.0 to 5.6; no growth occurs at pH 3.6 or at pH 7.3. *Relation to oxygen:* facultative anaerobe. *Resistance to heat:* survives for 15 min. at 60–65° in unhopped beer. *Resistance to ethanol:* growth is delayed in beer containing 8 % (v/v) of ethanol and fails in presence of 10 %.

Biochemical characters

Catalase reaction: negative. *Nitrate* not reduced to nitrite. *Indole* not formed. *Acetylmethylcarbinol* not formed. *Gelatin* not liquefied. *Litmus milk:* no change observable.

Utilization of carbohydrates. In a casein double-digest supplemented with yeast autolysate growth occurred and acid was formed from: arabinose, xylose, glucose, fructose, mannose, sucrose or maltose; gas formation did not occur. The organism showed slight growth when mannitol, salicin or inulin was used as carbon source but acid was not detected in these cultures.

The acid produced from glucose. The behaviour of HH 5 in the glucose medium was heterofermentative, yielding lactic and acetic acids in the molecular ratio 100 : 16.

CLASSIFICATION

The ability of the organism to ferment certain carbohydrates and the fact that it is a facultative anaerobe which is catalase-negative, does not reduce nitrate to nitrite and is non-motile, non-spore forming and Gram-positive, places it in the tribe Lactobacilleae Winslow *et al.* of the family Lactobacteriaceae Orla-Jensen. The additional features, a rod-shaped cell which produces lactic acid from glucose, allow the organism to be classed as a species of *Lactobacillus*.

The description of this organism has been compared with such information concerning *Lactobacillus* species as is available in the literature (Pederson, 1938; Orla-Jensen, 1942; *Bergey's Manual*, 1948; Shimwell, 1949). On this evidence the only species which is somewhat similar to HH 5 is *L. pastorianus* van Laer. However, the following differences are to be observed between the two organisms: cells of *L. pastorianus* appear as rods 5–10 μ . in length and even up to 35 μ . as filamentous forms, whereas HH 5 is about 1.1 μ . in length. Maximum temperature for growth of *L. pastorianus* is 38° with optimum range 29–33°. HH 5 has the low optimum 22–23° and fails to grow at 30°. Similarly, in its pH range HH 5 is markedly restricted in growth below pH 4.2 and fails to grow above pH 6.9, whereas *L. pastorianus* can be culti-

vated over the pH range 3.4–8.0. Finally, *L. pastorianus* ferments galactose, lactose, raffinose and salicin, none of which is attacked by the new organism. It is concluded, therefore, that HH5 represents a species of *Lactobacillus* hitherto undescribed. Since one of the main features of HH5 is its comparatively low optimum temperature and the low maximum temperature at which growth can occur, it has been named *L. frigidus*.

***Lactobacillus frigidus* n.sp.**

Cells short thick rods, $0.7 \times 1.1 \mu$. Non-motile. No endospores, capsules or flagella. Gram-positive. Colonies on beer-gelatin 1.0–1.5 mm. diameter, entire, convex, colourless.

Facultative anaerobe. Optimum temperature, 22–23°. Optimum hydrogen ion concentration, pH 5.0–5.6. Rapid growth in unhopped beer, unhopped malt wort, glucose nutrient broth and in a casein double-digest with added yeast autolysate. No growth in milk.

Ferments arabinose, xylose, glucose, fructose, mannose, sucrose and maltose, in all cases with production of acid but not of gas. Slight growth on mannitol, salicin and inulin but acid not detected. Heterofermentative on glucose.

Isolated from a sample of brewery 'top-yeast' (*Saccharomyces cerevisiae*), Manchester, England, February 1950. Subcultures from the type culture have been deposited at the National Collection of Industrial Bacteria (The Chemical Laboratory, D.S.I.R., Teddington, Middlesex) as Culture no. NCIB8518, at the National Institute for Research in Dairying, Shinfield, Reading, and at the Laboratories of the Brewing Industry Research Foundation, Nutfield, Surrey. The authors have been informed that subcultures from the NCIB have been sent to the American Collection of Type Cultures.

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(Received 15 October 1952)

SHILO, M. & ASCHNER, M. (1953). *J. gen. Microbiol.* 8, 333-343.

Factors governing the Toxicity of Cultures containing the Phytoflagellate *Prymnesium parvum* Carter

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SUMMARY: Methods for the laboratory maintenance and purification of cultures of *Prymnesium parvum* are detailed. The upper limit of temperature for the growth of the organism is 30°. Light exerts an augmenting effect on the production of the toxin by *Pr. parvum*. Rapid and economical procedures for the bioassay of the toxin are based on the use of *Gambusia* minnows or tadpoles. Cell-free centrifugates of the cultures contain heat-labile toxic material which is non-diffusible through cellophan, sensitive to oxidizing agents, and reversibly inactivated by mild acidity. The toxin is rapidly inactivated by ubiquitous bacterial species (*Bacillus subtilis* and *Proteus vulgaris*). Charcoals, clays and calcium sulphate are efficient adsorbents of the toxic material. The concentration levels of toxin in cultures of *Prymnesium parvum* appear to reflect a dynamic equilibrium between toxin destruction and production; a similar equilibrium may prevail in nature.

A phytoflagellate toxin was first implicated in an occurrence of mass mortality among fish in Holland by Liebert & Deerns (1920). Subsequent study of a similar occurrence in Denmark by Otterstroem & Steeman-Nielsen (1939) identified the responsible organism as *Prymnesium parvum* Carter, and confirmed that the toxic effect was caused by an extracellular thermolabile toxin. Reich & Aschner (1947) described the widespread occurrence of this organism in Israel and proposed measures for its control in fishponds.

The present communication describes methods for the maintenance and purification of laboratory cultures of *Prymnesium* spp. and procedures for the bioassay of the toxin. An analysis of the effects of certain environmental factors on the production and persistence of the toxin in pond water has been attempted. The experiments show the extracellular occurrence of the prymnesium toxin and suggest that it may be a protein.

EXPERIMENTAL

Bioassay of the toxin of Prymnesium parvum

Estimations of toxin content of culture fluids and of pond water were made by using *Gambusia* minnows or tadpoles of *Rana* or *Bufo* as the test organism. These species were readily available and could be handled in relatively small volumes of fluid. The tests are carried out as follows. For tadpole tests, five animals are immersed in 5 ml. volumes in 15 mm. diam. test-tubes. Cell-free centrifugates of the toxic samples are used for the assay. The dilution medium was sodium chloride solution (0.16%), 9 volumes + Sorensen's phosphate

buffer pH 8, 1 volume, the mixture being adjusted to final pH value of 7.5–8.5; temperature of test 15–25°. Bacterial contamination was prevented with streptomycin (1 mg./ml.). Assays were based on direct determinations of the minimum lethal dose or on observations of the time of onset of toxic reactions, particularly the loss of equilibrium sense in minnows (equilibrium-loss time), or of tail curvature in tadpoles (see Pl. 1). As the *Gambusia* minnow is the more sensitive test organism, it was preferred for work with dilute toxin. The response is, however, observed sooner in the tadpole, and this is therefore the preferable species for rapid assay work.

A prymnesium toxin unit (T.U.) is defined as the minimum amount of toxin/20 ml. which causes death of *Gambusia* minnows within 12 hr. in standard test conditions, specified for the *Gambusia* test as two minnows about 1.5 cm. long immersed together in 20 ml. test fluid in a 50 ml. beaker 40 mm. diameter. It should be noted that 1 T.U. in the standard volume of 20 ml. will kill more than the two *Gambusia* minnows, but twofold dilution of the fluid renders it non-toxic even to one minnow. The response observed in these conditions appears to depend on the toxin concentration rather than on the absolute amount of toxin present.

The relationship between toxin concentration and the equilibrium-loss time in minnows is shown in Fig. 1. The relationship between the minimum lethal concentration (M.L.C.) and the equilibrium-loss time was constant in all the toxin solutions tested. The equilibrium-loss time decreased as the amount of toxin increased above 1 T.U., and approached a constant (15–30 min.) at 5 T.U./20 ml. or more. Above 2 T.U./20 ml., the plot of the reciprocal of toxicity against the equilibrium-loss time deviates only slightly from a straight line. The loss of equilibrium sense was always followed by the death of the *Gambusia*. The minimal lethal concentration and the minimal concentration causing the loss of the equilibrium sense are therefore identical. Titrations of the equilibrium-loss time could be converted into T.U. by reference to Fig. 1. It is apparent that whereas direct determination of M.L.C. could only be completed in 24 hr., the observations of equilibrium-loss time afford reliable estimates of toxin concentration within 1 hr., the assay becoming increasingly sensitive and accurate as the equilibrium-loss time is allowed to increase beyond 1 hr.

The relationship of toxin concentration to time of showing tail curvature (curvature-time) in tadpoles is illustrated in Fig. 2. It is similar in general to that of equilibrium-loss time in *Gambusia* minnows to toxin concentration (cf. Fig. 1). The tadpole requires more toxin for a visible response, the minimum effective dose being 2.2 T.U./20 ml. As the toxin dose increased the curvature-time decreased at a decreasing rate and became constant above 50 T.U./20 ml. at a value near 8 min. Curvature-times of 15–30 min. were satisfactory for assay. Curvature response was always followed by the death of the tadpole.

The ratio of the minimum dose causing equilibrium loss to that causing tail curvature response was found to be 2.2 within a fluctuation limit of 10% in ten water samples from different natural sources. The constancy of ratio suggests that the equilibrium-loss in minnows and curvature response in

tadpoles are caused by the same toxin, though in this connexion it should be noted that tail curvature in tadpoles can also be elicited by other agents, e.g. D.D.T. (Schreiman & Rugh, 1949).

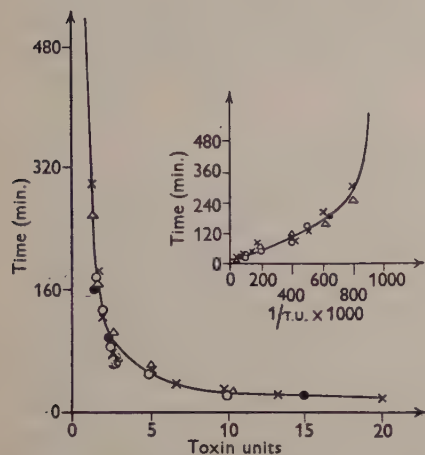


Fig. 1

Fig. 1. The relationship between toxin concentration and equilibrium-loss time in *Gambusia* minnows. Toxicity expressed in toxin units (T.U.)/20 ml. of immersion fluid was determined on graded dilutions of cell-free supernatant fluid of toxic *prymnesium* cultures. Different symbols represent different batches of toxin.

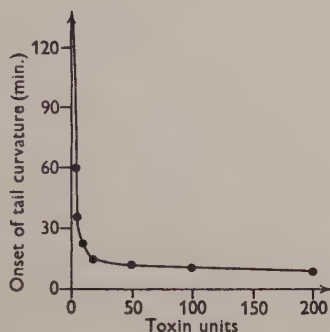


Fig. 2

Fig. 2. The relationship between toxin concentration and tail-curvature time in tadpoles. Toxicity tests were made on young *Rana* tadpoles. Values plotted represent averaged results of experiments with three toxin preparations. Toxin concentrations were converted to standard T.U. by multiplying the tadpole M.L.C. by the factor 2.2 which relates M.L.C. of minnows to that of tadpoles.

Preparation and storage of cell-free toxin solution

Liebert & Deerns (1920) showed that toxic water samples subjected to moderate heating may kill fish even though they no longer contain viable phytoflagellates. Otterstroem & Nielsen (1939) observed that centrifugation of pond water which resulted in a marked diminution of the flagellate concentration did not render the sample non-toxic and suggested that the toxic principle was extracellular. We found that centrifugation at 10,000 r.p.m. for 15 min. in the Servall angle centrifuge gives a cell-free toxin solution. Benzylpenicillin (500–1000 units/ml.) plus streptomycin (0.5–1.0 mg./ml.) were added to the toxin solutions to prevent bacterial contamination. This cell-free fluid was as toxic as the original cell suspension and could be stored without marked loss of potency in the refrigerator for a period up to 4 weeks.

Microscopical examination of cultures and determination of population densities

Living organisms were examined in hanging-drop preparations. For permanent preparations, the hanging drop was placed above iodine crystals, fixation being effected by the iodine vapour. The drops were spread out in

a thin film, dried, and stained for 1 sec. with undiluted Gram's gentian violet.

The suspensions of organisms were counted in a blood counting chamber which contained a small crystal of iodine in the overflow channel. Fixation with iodine preserved the shape of the rather fragile organisms of *Pr. parvum* better than other fixatives and made it easy to see the third flagellum, which is somewhat difficult to observe in life and which serves as the principal identifying characteristic of the genus.

Maintenance and purification of cultures

We used water from brackish fishponds in which the concentration of *Pr. parvum* varied from 10,000 to 5000,000 organisms/ml. Samples were kept at winter room temperature (12–15°) in the laboratory in a northern window or under a 40 W. daylight lamp. Tall cylindrical glass vessels or test tubes half-filled with medium served as culture vessels. Under these conditions the *Pr. parvum* multiplied and maintained toxicity for long periods, whereas most of the other algae present usually diminished in number until they could no longer be detected by direct microscopic examination. The persistence of low number of algal forms which were not *Prymnesium* spp. was demonstrated by adding ammonium sulphate in a concentration lytic to *Prymnesium* spp. or by heating at 35° for short periods; this was followed by reappearance of other algal species in the cultures.

The medium was pond water of suitable salinity (about 1000 mg. Cl/l.) which had been allowed to undergo self-purification in the dark, Chu's medium (1942), or sea water which had been diluted 1:10 with tap water. The pH values of these media ranged from 7.5 to 9.0. The prymnesium cultures were grown with massive transfer (1 volume of inoculum in 2–5 volumes of medium) at intervals of 2–4 weeks.

Typical growth is summarized in Table 1. Marked increases in the final prymnesium counts followed the incorporation of fish peptone or egg yolk (Table 2). Routine addition of these supplements was held to be undesirable, however, since they also promoted the growth of non-prymnesium members of the population, especially bacteria. Excessive proliferation of bacteria may lead to disappearance of *Pr. parvum* from the culture.

Temperatures higher than 30° were markedly inhibitory; exposure to 35° for intervals as short as 5 min. was sufficient to cause death. Caution was thus required when cultures were exposed to direct sunlight, and cooling of the cultures was necessary. *Pr. parvum* survives at temperatures as low as 2° for many days. The seasonal fluctuations of *Pr. parvum* density in ponds throughout Israel correspond broadly with these temperature requirements of the organism, the density rising markedly in winter and falling almost to nil at the beginning of summer when the pond temperatures approach 30°.

Adequate illumination of the cultures was important for cell proliferation and maintenance of culture toxicity. Light affected toxicity more quickly than cell number. The experiment in Table 3 shows the specific effect of light on the toxicity as distinct from its effect on cell proliferation. In cultures transferred in the laboratory under standard conditions there are generally

Table 1. *The multiplication of Prymnesium parvum in different nutrient media*

Inoculation of the nutrient media was by massive transfer of *Prymnesium parvum* after two previous transfers through the same media. The inoculum in these experiments was 1/3-1/10 of the total volume of the medium. Cultures were kept in 1 l. cylindrical containers at room temperature in diffuse northern light (1000-1500 lux).

Medium	Prymnesium counts (organisms/ μ l.) on successive days				
	0	1	2	4	8
Pond water:*					
Culture 1	120	280	500	970	1050
Culture 2	130	340	1070	1090	1660
Culture 3	370	830	—	1360	—
Tap water containing 7% sea water:					
Culture 4	230	—	250	410	500
Culture 5	600	—	—	1200	1500
Chu medium no. 10† containing 7% sea water:					
Culture 4	230	180	580	900	1400
Culture 3	300	660	—	1200	—

* Salinity of approx. 1000 mg. Cl/l.

† As described by Chu (1942).

Table 2. *Influence of nutrients added to pond water on prymnesium counts*

Pond-water cultures containing equilibrium populations of prymnesium were divided into similar glass containers and kept at room temperature in northern diffuse light with nutrient supplements as described below.

Nutrient supplement	Period of cultivation (days)			
	0	4	14	18
Prymnesium counts (organisms/ μ l.)				
Egg yolk: 0	390	500	400	530
1: 1,100	350	650	2,940	4,000
1: 5,000	340	480	1,230	1,700
1: 10,000	350	410	700	800
1: 50,000	390	280	400	420
1: 100,000	360	370	250	480
Fish peptone:* 0	820	860	900	—
1: 500	820	2050	2,300	—
1: 1,000	920	1910	2,470	—
1: 5,000	740	—	900	—
1: 10,000	610	—	690	—
0	120	140	150	—
1: 1,000	100	480	1,250	—

* As prepared by Snieszko, Griffin & Friddle (1950).

1 to 4 T.U./10⁶ organisms, the concentration of the toxin 2-6 days after transfer being c. 1 T.U./ml. of culture.

The cultures also contained protozoa and bacteria. The relationship between the different species is not well understood. As is shown in Table 4, *Pr. parvum* possess a relatively high degree of resistance to several antibiotics, and

Table 3. *Influence of light on cell density and toxicity of prymnesium cultures*

In this experiment a sample of non-toxic pond water was dispensed into three cylindrical vessels which were placed at room temperature under different lighting conditions as indicated below. Toxicity was tested with *Gambusia minnows*. Illumination was measured with the help of an exposure-meter and expresses average intensity in each condition at noon.

Illumination of culture	Relative light intensity	Age of culture								
		0 hr.			24 hr.			72 hr.		
		Count of prymnesium (organism/ μ l.)	Toxicity (units/20 ml.)	T.U./10 ⁶ cells	Count of prymnesium (organism/ μ l.)	Toxicity (units/20 ml.)	T.U./10 ⁶ cells	Count of prymnesium (organism/ μ l.)	Toxicity (units/20 ml.)	T.U./10 ⁶ cells
Diffuse day-light	12	250	<1	<0.2	260	5	1	420	10	1.2
Fluorescent light*	9	250	<1	<0.2	350	<1	<0.3	240	4	0.8
Laboratory interior	7	250	<1	<0.2	260	<1	<0.2	140	<1	<0.3

* 40 W. lamp operated for 12 hr./day.

Table 4. *Survival of prymnesium in cultures containing antibacterial concentrations of selected antibiotics*

Pond-water cultures with specified antibiotic were kept in northern diffuse light at room temperatures (22°). The prymnesium counts are indicated as follows:

++++ 500-1000/ μ l.; +++ 250-500/ μ l.; ++ 50-250/ μ l.; + 10-50/ μ l.; - <10/ μ l.

Antibiotic added	Time of assay after addition of antibiotic (days)	Concentration of antibiotic (mg./ml.)								
		6.5	3.3	2.0	1.0	0.5	0.25	0.1	0.05	0.01
		Survival of prymnesium								
Penicillin G*	1	++	++++	++++	++++	++++	++++	++++	++++	++++
	3	—	++++	++++	++++	++++	++++	++++	++++	++++
	7	—	++++	++++	++++	++++	++++	++++	++++	++++
Streptomycin†	1	.	.	++++	++++	++++	++++	++++	++++	++++
	3	.	.	+	++++	++++	++++	++++	++++	++++
	7	.	.	—	++++	++++	++++	++++	++++	++++
Terramycin	1	.	.	—	—	—	+	+	++	++++
	3	.	.	—	—	—	—	+	+	+++
	7	.	.	—	—	—	—	—	+	++
Chloramphenicol	1	.	.	—	—	—	++	++	+++	++++
	3	.	.	—	—	—	—	—	++	++++
	7	.	.	—	—	—	—	—	—	++++
Aureomycin	1	.	.	—	—	—	—	—	++	+++
	3	.	.	—	—	—	—	—	—	++
	7	.	.	—	—	—	—	—	—	++

* 1430 units/mg.

† Similar results were also observed with dihydrostreptomycin.

advantage may be taken of this to purify cultures. The *Pr. parvum* survived and even multiplied in the presence of bactericidal concentration of streptomycin and penicillin. When 0.1 ml. samples of culture fluid containing these antibiotics were inoculated into nutrient broth, brain and heart infusion, and into glucose agar media, they often failed to show bacterial growth within 24-48 hr. after the addition of the antibiotics. Cultures of *Pr. parvum* which

were bacteria-free by this criterion were carried in an antibiotic-free medium for several transfers. Such cultures tended to die after 2-3 weeks. The causes of the decline are being studied.

Population density of Prymnesium parvum in pond-water samples and laboratory cultures; relationship to the toxicity of the culture fluids

Counts of *Pr. parvum* and toxicity assays of pond-water samples taken from selected locations are shown in Table 5; the counts are arranged in order of size.

Table 5. *Population of Prymnesium spp. and toxicity of fishponds*

Location and serial number of pond		Date of sample	Prymnesium count (organisms/ μ l.)	Toxicity (units/20 ml.)
Sdeh Nachum	4	25. xi. 46	1080	1.0
	7	28. x. 46	960	1.0
Messiloth	6	10. xii. 46	870	1.0
	6	15. xii. 46	600	1.0*
	12	11. v. 49	550	1.0
	12	17. v. 49	400	1.0*
Tel Amal	Z	20. xi. 50	300	1.0
	Z	26. xi. 50	300	1.0*
Messiloth	6	6. vi. 52	240	10-20
Sdeh Nachum	7	26. viii. 48	60	1.0*
Tel Amal		1. iii. 47	50	1.0*

1.0* indicates toxin concentration which killed carp, i.e. = 1.3 T.U.

As Reich & Aschner (1947) showed there is no simple relationship between the *Pr. parvum* count and the toxicity of the pond-water sample. In fact, very high prymnesium counts are sometimes associated with absence of toxicity (e.g. 7×10^6 organisms/ml.; < 0.05 T.U./ml.). On the other hand, several highly toxic samples of pond water gave very low prymnesium counts (5×10^4 organisms/ml.; > 0.5 T.U./ml.). The wide variation of the toxicity:count ratio might be due to the occurrence of variants of *Pr. parvum* or to differential effects of environmental factors on the production and destruction of the toxin. The notion that the low toxicity:count ratios are characteristic for non-toxigenic variants could be ruled out by the observation that pond-water samples of low or undetectable toxicity and a high count for prymnesium regularly yield highly toxic cultures when maintained in the laboratory under standard conditions for 2-3 days. During this interval the increase in the flagellate population is less than twofold, and it seems unlikely that a process of natural selection favouring a toxigenic variant is responsible for the increase in toxicity.

When the population density of laboratory cultures was increased by growth-promoting supplements to numbers in excess of 5×10^6 flagellates/ml., an inverse relationship between cell count and toxicity was sometimes observed. Dilution of such dense cultures with sea water or pond water rapidly re-established the normal toxicity:count ratio. An example of such an experiment is given in Table 6. Cultures of high cell count have a deep brown colour

and considerable turbidity. It remains to be ascertained to what extent the decrease in toxicity which is associated with excessive cell proliferation may be due to the inadequate illumination of the interior of the culture.

Table 6. *Effect of dilution on toxicity of dense prymnesium cultures*

A static culture of *Prymnesium parvum* which had been fortified with a soil supplement and contained 7.3×10^6 prymnesium organisms/ml. was diluted in the proportions shown below with 1:10 strength sea water and held for 24 hr. in cylindrical vessels at room temperature under a 40 W. daylight lamp.

Dilution of culture with 1:10 strength sea water	Organisms $\times 10^6$ /ml.		T.U./20 ml.	
	0 hr.	24 hr.	0 hr.	24 hr.
1:0	7.30	7.30	<1.0	<1
1:4	1.46	1.66	<1.0	2.5
1:0	0.73	0.90	<1.0	5.0

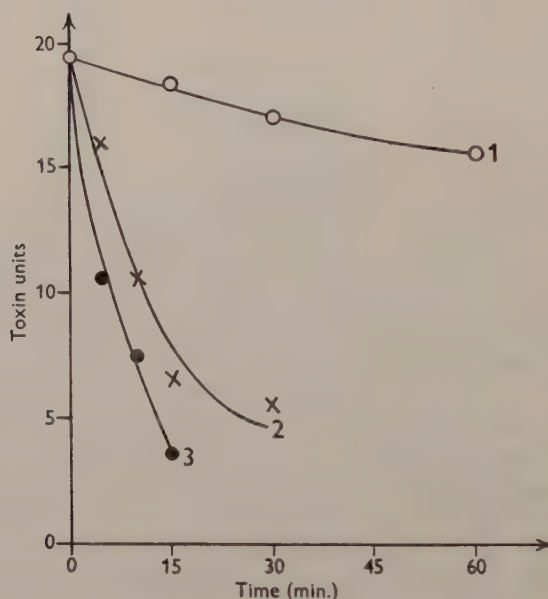


Fig. 3. The effect of temperature on prymnesium toxin. The toxicity expressed in T.U./20 ml. was tested with *Gambusia*. Curves 1, 2 and 3 show effects of exposure to 62, 80 and 97° respectively.

Observations on general properties of the toxin of Prymnesium parvum cultures

Effect of temperature. At 97 and at 80°, the toxicity of the culture supernatant fluid declined rapidly; at 62° the decline was relatively slow. Thermal inactivation became progressively slower as the concentration of the toxin fell (Fig. 3). At room temperature and at 4° there was no detectable loss of toxicity for at least 7 days, provided that bacterial growth did not occur and oxidation was prevented.

Diffusibility. Solutions of toxin in cellophan tubing showed no significant fall in titre when suspended in frequently renewed distilled water or in isotonic sodium chloride solution (0.16 %) for 12–24 hr. at 4°.

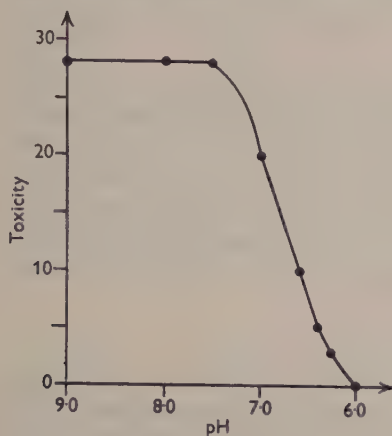


Fig. 4

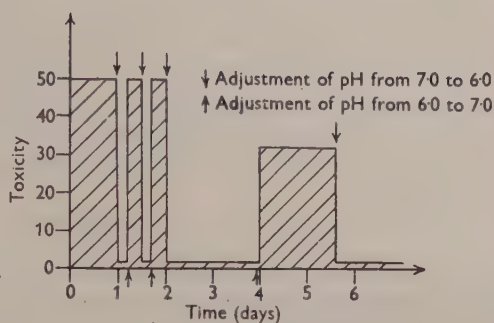


Fig. 5

Fig. 4. Influence of pH on activity of toxin. The toxin solution contained 28 T.U./20 ml. The pH was adjusted with phosphate buffer 0.02 M in the range pH 6.0–8.0 and with borate buffer (Clark) at a final concentration of 1:10 in the range pH 7.8–9.0. The toxicity was measured on *Gambusia* 1 hr. after the adjustment of the pH of the toxin solution. Buffer solutions containing 10 % sea water of the same pH and concentration were used for dilution.

Fig. 5. Reversible inactivation of toxin at acid pH values. Toxicity is expressed as the reciprocal of the equilibrium-loss time in minutes. pH was adjusted as indicated by addition of 0.5 N-hydrochloric acid or sodium hydroxide.

Effect of pH value. The influence of the pH value on toxicity is illustrated in Fig. 4. Toxicity was independent of pH value within the range pH 7.5–9.0. The toxicity decreased rapidly at pH values below 7.5, and was zero at pH 6.0. At moderate acidities, the inactivating effect of hydrogen ion was almost completely reversible for at least several days (Fig. 5). This behaviour resembles that of different enzyme proteins. Assuming that the variation of the pH value is modifying the nature of the toxin, it would follow that the toxin possibly possesses a functional group of pK near 6–7. However, it should not be overlooked that the variation of the pH value might also be affecting the susceptibility of the test animal.

Oxidizing agents. Toxicity declined when oxygen was bubbled through a solution of toxin; air produced the same result though somewhat more slowly. Potassium permanganate (2×10^{-4} M) or sodium hypochlorite (2.0 p.p.m.) added to culture supernatant fluid containing 0.5 T.U./ml. immediately destroyed toxicity.

Adsorbents. A wide variety of adsorbents removed the toxic material from culture supernatant fluid. At room temperature, the toxicity of 50 ml. containing 2.5 T.U. was completely removed within 5 min. by 2–5 g. of kaolin, Norit A (acid washed), activated charcoal, or calcium sulphate ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$).

Under the same conditions, Decalso, calcium carbonate, kieselguhr, Fuller's earth and activated alumina (Eimer & Amend) removed little or no toxin. Highly toxic cultures were rapidly detoxified by stirring with pond-bottom soils. When such cultures are then allowed to remain in contact with the soil, they continue non-toxic for periods up to a fortnight in conditions which are otherwise favourable to toxin production.

Destruction of toxin by micro-organisms. A destructive action of micro-organisms on the toxin was suggested by the observation that the maintenance of the toxicity of stored toxin solution at all temperatures compatible with bacterial growth was conditional on the addition of suitable antibiotics to the toxin sample. Washed suspensions of *Proteus vulgaris* and of *Bacillus subtilis* decreased the potency of prymnesium toxic culture supernatant fluid by at least 50 % in 1 hr., whereas *Bacterium coli* was almost without effect under the same conditions.

DISCUSSION

The prymnesium toxin appears to be a substance of high molecular weight with some of the properties of a protein. It is readily differentiated from other algal toxins, notably those found in marine dinoflagellates (Sommer *et al.* 1948*a, b*; Connell & Gross, 1950) and in *Microcystis* (Prescott, 1948; Ashworth & Mason, 1946; Shelubsky, 1951) which are intracellular constituents of cells and not excreted into the medium. Some explanation can now be offered of the puzzling lack of correlation between the population density of *Prymnesium parvum* and the toxicity of the pond water. The pronounced lability of the prymnesium toxin to oxidizing agents, its ready adsorption by bottom soil and its rapid destruction by micro-organisms may explain non-toxic pond waters. Light, by augmenting toxin production, works in the opposite direction, and is probably one of many factors which regulate toxin production in nature. The concentration of toxin observed represents, therefore, a dynamic equilibrium between production and destruction. The dramatic increases in toxicity which may be observed in fish ponds with stable prymnesium populations can thus represent either the withdrawal of a toxin-removing agency or an enhancement of a factor concerned in toxin production.

This work was supported in part by a grant from the Fish Breeders Association of Israel. The co-operation of the Department for Fisheries of the Ministry of Agriculture of the Government of Israel is also gratefully acknowledged. We are grateful to Mrs Miriam Shilo for her contributions to the experiments on the purification of the prymnesium cultures, to Mr S. Sarig for help in the field work, and to Dr S. Hestrin for valuable suggestions.

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Fig. 1

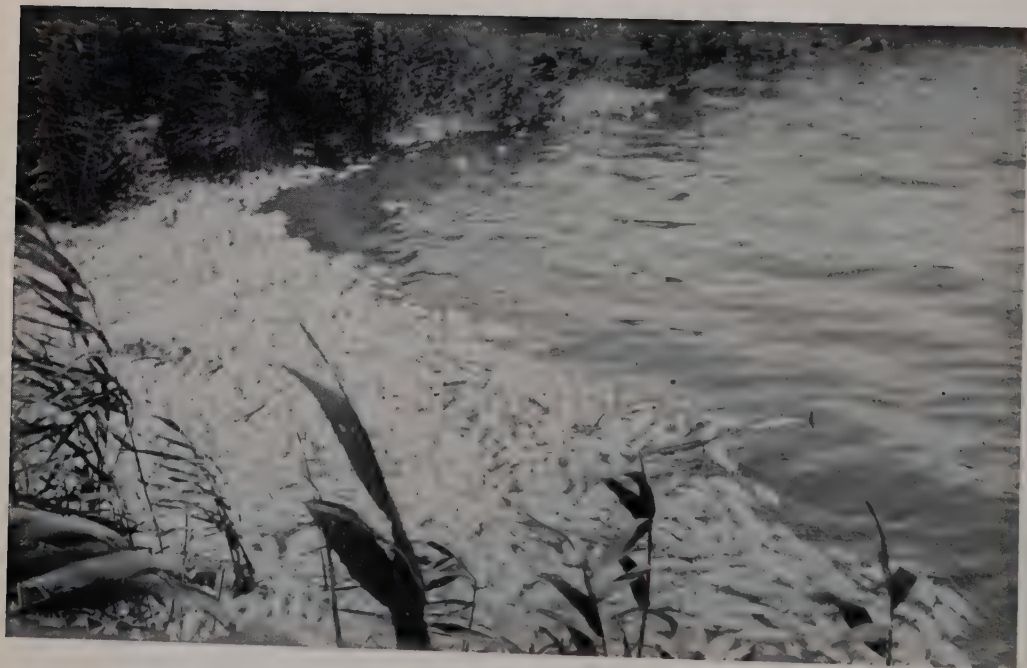


Fig. 2

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EXPLANATION OF PLATE

Fig. 1. Tadpoles of *Rana* sp. after 15 min. in toxin solution. Typical curvature of the tail is the first visible effect of the intoxication.

Fig. 2. Mass-mortality of fish in a carp-breeding pond caused by toxin of *Prymnesium parvum*.

(Received 5 August 1952)

The Oxidation of Inorganic Compounds of Sulphur by various Sulphur Bacteria

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SUMMARY: Cultures of *Thiobacillus thiooxidans*, *Th. thioparus*, *Th. novellus*, *Thiobacillus B* (Waksman, 1922a), strains 'T' and 'K' (Trautwein, 1921) and *Th. concretivorus*, *Thiobacillus X* and the 'M' strains, organisms isolated from concrete (Parker, 1945, 1947), were examined to elucidate the mode of oxidation and to establish the identity of the organisms recently isolated from corroded concrete.

Thiosulphate was oxidized by all these bacteria. *Th. thiooxidans*, *Th. concretivorus* and *Thiobacillus X* first converted it to tetrathionate and sulphate and then oxidized the tetrathionate to sulphate and free sulphuric acid. *Thiobacillus X* differed from the other two in that, owing to a lesser acid tolerance, some tetrathionate was found in the final products of oxidation. *Th. thioparus* converted thiosulphate to sulphate and sulphur, followed by partial oxidation of the sulphur to sulphuric acid. *Th. novellus* produced sulphate and sulphuric acid. *Thiobacillus B*, the 'T' and 'K' strains and the 'M' strains formed sulphate and tetrathionate with temporary increase in pH value; only *Thiobacillus X* oxidized tetrathionate, yielding sulphate and sulphuric acid.

Elementary sulphur was oxidized by *Th. thiooxidans*, *Th. concretivorus*, *Thiobacillus X* and *Th. thioparus*; the rates of oxidation decreased in that order, and the only product was sulphuric acid.

Hydrogen sulphide was oxidized only at low concentrations and only by *Th. concretivorus* and *Thiobacillus X*; sulphuric acid was the end-product, and elementary sulphur may have been an intermediate.

Thiobacillus X differed from *Th. thiooxidans* in pH range for growth and from *Th. thioparus* in its method of oxidation of thiosulphate, tetrathionate and H_2S . It appeared to fit earlier descriptions of *Th. thioparus* by Nathansohn (1902) and Beijerinck (1904) more closely than the strain described as *Th. thioparus* by Starkey (1934a). The 'M' strains were similar to *Thiobacillus B* and the 'T' and 'K' strains of Trautwein.

Species of micro-organisms capable of oxidizing inorganic sulphur compounds fall into three main groups: those which oxidize hydrogen sulphide autotrophically and deposit sulphur intra- or extracellularly, including members of the Beggiatoaceae, Thiorhodaceae and Chlorobacteriaceae families of the classification of *Bergey's Manual* (1948); members of the genus *Thiobacillus* which oxidize a wide variety of sulphur compounds; various heterotrophic organisms such as *Pseudomonas* spp. which appear to have a much more limited ability for oxidation.

Difficulty in isolating pure cultures of members of the first group has retarded the elucidation of their metabolic activities. However, pure culture studies of the Thiorhodaciae and Chlorobacteriaceae by van Niel (1931) have thrown considerable light on the mechanism of photosynthesis by these organisms. Detailed studies of the oxidative ability of organisms of the second and third

groups were made by Starkey (1925, 1934*b*, 1935*a*). More recently, the work of Umbreit and colleagues (Vogler, 1942; Vogler & Umbreit, 1942; LePage & Umbreit, 1943) with *Thiobacillus thiooxidans*, has revealed something of the nature of the chemosynthetic process.

However, the ability of several species of the second and third groups to oxidize some forms of sulphur remains uncertain, and the exact course of the oxidation is still open to doubt. To help resolve these points and to establish the identity of species of sulphur bacteria recently isolated from corroded concrete (Parker, 1945, 1947) an examination of several species of bacteria able to oxidize inorganic sulphur compounds has been made and some of Starkey's work has been repeated in greater detail. Three types of organism isolated from concrete were examined: the autotrophic *Th. concretivorus* (Parker, 1945); the *Th. thioparus*-like autotrophic type (Parker, 1947) here referred to as *Thiobacillus X*; a miscellaneous collection of heterotrophic types (Parker, 1947) here referred to as the 'M' strains; strains of *Th. thiooxidans*, *Th. thioparus*, *Th. novellus*, *Thiobacillus B* (Waksman); strains 'T' and 'K' of Trautwein. These latter were kindly supplied by Dr R. L. Starkey and, except for *Th. thioparus*, were subcultures of those originally isolated. The strain of *Th. thioparus* was a subculture of an organism isolated by Starkey (1934*a*) and considered by that author to be identical with the original strain isolated by Nathansohn (1902) and named by Beijerinck (1904).

METHODS

Media. The constituents of the basal media used are shown in Table 1. To study the oxidation of the various inorganic sulphur compounds, appropriate alterations were made in the sulphur compound added.

Table 1. *Composition of media used in study of oxidation by various sulphur bacteria*

	Medium					
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
	(g./l.)					
(NH ₄) ₂ SO ₄	0.1	0.1	0.1	—	—	—
NH ₄ Cl	—	—	—	0.1	0.1	0.1
K ₂ HPO ₄	2.0	4.0	2.0	0.2	—	—
KH ₂ PO ₄	—	4.0	—	—	3.0	3.0
MgSO ₄ ·7H ₂ O	0.1	0.1	0.1	—	—	—
MgCl ₂ ·6H ₂ O	—	—	—	2.5	0.1	0.1
CaCl ₂	0.1	0.1	0.1	—	0.1	0.1
FeCl ₃ ·6H ₂ O	0.02	0.02	0.02	—	—	—
MnSO ₄ ·4H ₂ O	0.02	0.02	0.02	—	—	—
NaHCO ₃	—	—	—	1.0	—	—
CaCO ₃	—	—	Excess	—	—	—
Sulphur	—	—	—	—	—	10.0
Na ₂ S ₂ O ₃ ·5H ₂ O	10.0	10.0	10.0	5.0	5.0	—
pH value	8.5	6.6	9.2	9.1	4.2	4.2

Media 1 and 2 are those used by Starkey (1934*a*); medium 3 is similar to his no. 3. Media 4, 5 and 6 are similar to those used by Beijerinck (1904).

The rate of oxidation could be varied by altering the ratio of surface area exposed to air to volume of culture medium, so appropriate volumes of medium were placed in Erlenmeyer flasks depending on the speed of reaction desired. In general, 100 ml. medium was used in a 300 ml. Erlenmeyer flask. Separate flasks were taken for analysis at intervals. In some experiments, however, 1.5 l. medium was used in a 2 l. flask and samples were taken at intervals from it.

Media containing thiosulphate were steamed in bulk on three successive days and then distributed aseptically in 100 ml. lots into sterile 300 ml. Erlenmeyer flasks. Medium 6 was autoclaved in separate flasks and the sulphur was steamed in test tubes and then added to the medium.

Media containing tetrathionate were incubated in 50 ml. lots in 100 ml. Erlenmeyer flasks. Potassium tetrathionate was prepared in this laboratory as follows: Wackenroder's solution was prepared by passing H_2S through a saturated solution of sulphurous acid held just above 0° (Debus, 1888). When the reaction was completed sulphur was filtered off and the solution concentrated to sp.gr. 1.82 by evaporation on a water-bath. Potassium salts of the mixed thionic acids were then prepared by the addition of a saturated solution of potassium acetate to the concentrated Wackenroder's solution. The potassium salts were allowed to crystallize for 24 hr. and crystalline pentathionate and tetrathionate filtered off. The mixed crystalline mass was recrystallized from water slightly acidified with sulphuric acid and the potassium tetrathionate separated by hand picking typical crystals. It was possible in this way to separate material which appeared to be pure potassium tetrathionate; this was again recrystallized from 90% (v/w) ethanol. The final product was shown to be free from thionates other than tetrathionate by the analytical methods described below.

Inoculum. Suspensions of the various strains were obtained by washing the growth from an agar slope with 1–2 ml. sterile distilled water containing thiosulphate; 0.25 ml. of this suspension was used to inoculate each flask. With cultures which grew well on agar medium the number of organisms in the culture medium immediately after inoculation was about 10^6 /ml., while cultures which grew poorly on agar medium, such as *Th. novellus*, gave an initial viable count of about 10^3 /ml.

Incubation. Cultures were incubated at 28° , except for those grown in an atmosphere of hydrogen sulphide. Loss of water during prolonged incubation was minimized by using small incubators each containing a shallow vessel of water. When hydrogen sulphide oxidation was examined, the inoculated flasks were incubated at room temperature (about 18°) in a glass box, through which was passed air containing approximately 200 p.p.m. H_2S .

Analyses. Usually a whole flask was taken for analysis; when significant evaporation had occurred, the contents were first transferred to a standard flask and diluted with sterile distilled water to the original volume. The bacterial count was determined by serial tenfold dilution of 1 ml. medium in sterile distilled water and inoculation of the dilutions into appropriate culture medium. This procedure was only accurate to a power of 10, but was sufficiently

accurate to indicate the major changes in population during incubation. The pH values were determined electrometrically.

Cultures were filtered through asbestos in Gooch crucibles into dry flasks, to remove elementary sulphur. The sulphur adhering to the incubated flask was later removed and washed into the same crucible. Samples from the filtered culture were quickly measured and the analyses begun at once, as in some cases decomposition of the solution, shown by clouding, took place very soon after filtration.

Thiosulphate, polythionate, tri-, tetra- and pentathionate, and elementary sulphur were determined by the methods of Starkey (1935*a*). The term 'elementary sulphur' is used to describe any form of insoluble sulphur precipitated during growth. Sulphates were determined gravimetrically as BaSO_4 . *Sulphide* was determined by the methylene-blue method of Pomeroy (1936), except in media where it was inapplicable. In these cases the antimony sulphide method (*Standard Methods of Water Analysis*, American Public Health Association, 8th ed.) was used. *Sulphide* was determined as 'sulphide after acidification' without reference as to whether the sulphide was present as dissolved hydrogen sulphide, hydrosulphide, or polysulphide. The analytical results for the various forms of sulphur are recorded in the Tables as mg. S/l. culture medium.

RESULTS

Thiobacillus concretivorus

Thiosulphate oxidation. The method of oxidation of thiosulphate by this strain has been described (Parker, 1945). In the present investigation a more detailed examination of the course of reaction was made. Flasks of medium 5 were inoculated with a young culture and analysed at frequent intervals. Two different strains were examined, but, as they behaved similarly, the results with one strain only are shown (Table 2). The chemical changes fell into two

Table 2. *Oxidation of thiosulphate by Thiobacillus concretivorus*

Cultures in medium 5 were analysed at intervals after inoculation

Period of incubation (hr.)	pH value	Form of S				Viable count (organisms/ml.)
		$\text{S}_2\text{O}_3^{2-}$	$\text{S}_4\text{O}_4^{2-}$	SO_4^{2-}	S (element)	
		Concentration (mg.S/l.)				
0	4.4	1030	0	40	nt	10^6
42	4.8	1020	0	80	nt	10^6
114	4.4	800	200	110	nt	10^7
138	4.1	275	560	240	20	10^8
162	2.3	0	10	1000	60	10^7

nt = not tested.

stages: (1) during oxidation of the thiosulphate and before its complete disappearance, substantial quantities of polythionate were detected; (2) quantitative examination indicated that the polythionate was almost entirely tetrathionate. Tetrathionate persisted until all the thiosulphate had been

oxidized. Owing to the rapidity of oxidation, polythionates could only be detected over a period of 2 or 3 days. This transitory occurrence of polythionate was no doubt responsible for the failure to detect it in the previous work. During the conversion of thiosulphate to tetrathionate, there was very little change in pH value of the medium. There was a definite increase in sulphate and the bacterial count also increased. Very small amounts of elementary sulphur were formed.

After the complete disappearance of thiosulphate, the tetrathionate disappeared, this change being completed within 24 hr. It was accompanied by a further increase in sulphate concentration, a very marked decrease in pH value and the formation of a further small amount of elementary sulphur. The bacterial count remained high during this stage.

No further chemical change of any magnitude occurred after the complete oxidation of tetrathionate to sulphate, but the culture remained viable for at least a further 6 days. This was probably due to the presence of the small amounts of elementary sulphur which served as a further source of energy after thiosulphate and tetrathionate had disappeared.

Starkey (1925) found that the course of oxidation of thiosulphate by *Th. thiooxidans* was influenced by the thiosulphate concentration. The influence of concentration of thiosulphate on the course of oxidation was therefore examined with *Th. concretivorus*. Batches of medium 5, with thiosulphate

Table 3. *Effect of initial thiosulphate concentration on oxidation of thiosulphate by Thiobacillus concretivorus*

Cultures in medium 5 with various initial concentrations of $\text{Na}_2\text{S}_2\text{O}_3$ were analysed at intervals after inoculation.

Initial thiosulphate concentration (% Na ₂ S ₂ O ₃)	Period of incubation (hr.)	pH value	Form of S				Viable count (organisms/ ml.)
			S ₂ O ₃ ²⁻	S ₄ O ₆ ²⁻	SO ₄ ²⁻	S element	
			Concentration (mg. S/litre)				
1	0	4.6	2,580	0	30	0	10 ⁶
	42	4.2	2,510	40	nd	0	10 ⁶
	114	4.7	1,545	370	nd	10	10 ⁷
	162	2.6	0	870	nd	100	10 ⁷
	210	nd	0	20	nd	240	10 ⁷
	306	nd	0	0	2,590	10	10 ³
2	0	4.6	5,250	0	60	0	10 ⁶
	144	4.6	4,370	400	nd	60	10 ⁶
	240	4.6	3,250	940	nd	nd	nd
	264	3.3	180	680	nd	120	10 ⁷
	336	1.3	0	10	nd	380	10
	600	1.6	0	0	5,200	130	10
4	0	4.7	10,360	0	60	0	10 ⁶
	120	4.9	9,580	0	nd	60	10 ⁵
	288	4.9	8,490	0	nd	550	10 ⁷
	480	4.6	3,320	2,770	nd	nd	10 ⁶
	624	3.7	2,660	1,190	nd	1,010	10 ⁴
	840	1.8	0	0	8,770	1,290	0

nd = not determined.

concentrations of 1.0 % (w/v), 2.0 % (w/v) and 4.0 % (w/v) were inoculated and the reaction followed. Thiosulphate oxidation was not inhibited by 4 % (w/v) sodium thiosulphate, although the time required for complete oxidation increased with concentration of thiosulphate. The decrease in pH value was delayed with the higher concentrations of thiosulphate, though ultimately it fell to less than pH 2.0 with all concentrations. (Starkey noted a similar phenomenon with *Th. thiooxidans*.) Tetrathionate was formed during thiosulphate oxidation at each concentration but disappeared before the cultures became sterile. Elementary sulphur formation was observed at each concentration, and was greater the higher the concentration. At the end of the incubation, the sulphur was present predominantly as sulphate at each concentration.

Tetrathionate oxidation. The finding that tetrathionate was an intermediate product of thiosulphate oxidation by *Th. concretivorus* made it desirable to examine its ability to oxidize tetrathionate. Quite high concentrations of tetrathionate were produced as an intermediate from thiosulphate, but the tetrathionate so formed was quickly oxidized further to sulphate and free sulphuric acid.

Medium 5, in which 0.5 % (w/v) potassium tetrathionate replaced thiosulphate, was inoculated with this strain, but, despite repeated attempts, neither growth nor oxidation of the tetrathionate occurred. As growth, tetrathionate formation and oxidation occurred in medium 5 when potassium thiosulphate replaced sodium thiosulphate, the absence of growth with potassium tetrathionate cannot be attributed to an absence of sodium ions. The inability of this organism to oxidize tetrathionate, despite its destruction by cultures in thiosulphate medium, is hard to understand, and no satisfactory reconciliation of the two observations can be made at present.

Oxidation of elementary sulphur. The method of oxidation of elementary sulphur by this organism was studied by Parker (1945). The sole product of oxidation in the presence of excess sulphur was sulphuric acid which reached concentrations of over 5 % (w/v).

H₂S oxidation. Parker (1945) reported that this organism did not utilize H₂S when cultures were exposed to air containing 1 % (v/v) H₂S. In the present work the organism was inoculated into liquid mineral salt medium containing no oxidizable form of sulphur and incubated in air containing 200 p.p.m. H₂S; initial pH value of medium 4.5. The results of analyses during incubation are shown in Table 4.

Metabolism was indicated by the continuous viable count throughout the period of incubation and was accompanied by a rapid drop in pH value from 4.5 to 1.8 in the inoculated flasks. The increase in the total sulphur content of the inoculated compared with the sterile flasks shows that H₂S was utilized. The end-product of oxidation was mainly sulphuric acid, but the presence of a small amount of elementary sulphur towards the end of the incubation period in the inoculated flasks, and its absence from the controls, suggested that sulphur was first formed and then oxidized. Thiosulphates and polythionates were not detected at any stage of the incubation. The growth of the

organism in air containing 200 p.p.m. H_2S compared with its lack of growth in 1 % (v/v) H_2S suggests that the higher concentration was toxic.

Table 4. *Oxidation of H_2S by Thiobacillus concretivorus*

Cultures in medium 5 without thiosulphate, but with an atmosphere of air containing 200 p.p.m. H_2S were analysed at intervals after inoculation.

Period of incubation (days)		pH value	Form of S			Viable count (organisms/ml.)
			SO ₄ ^o	S (element)	Sulphide	
			Concentration (mg. S/l.)			
Inoculated culture	6	3.1	45	10	0	10 ⁷
	11	2.3	158	73	0	10 ⁷
	18	2.2	301	135	0	10 ⁴
	21	2.0	389	nd	0	10 ⁴
	29	1.8	481	405	0	10 ⁵
Sterile control	6	4.5	0	0	0.9	0
	11	4.4	0	0	0.6	0
	18	4.4	0	0	0.6	0
	21	4.4	0	0	0.6	0
	29	4.4	0	0	0	0

nd = not determined.

Thiobacillus thiooxidans

Thiosulphate oxidation. The route of oxidation of thiosulphate by this organism was described by Waksman & Starkey (1923). Since tetrathionate was detected during the oxidation of thiosulphate by *Th. concretivorus*, a more detailed examination of its oxidation by *Th. thiooxidans* was made.

Table 5. *Oxidation of thiosulphate by Thiobacillus thiooxidans*

Cultures in medium 5 were analysed at intervals after inoculation.

Period of incubation (hr.)	pH value	Form of S				Viable count (organisms/ml.)
		$S_2O_3^{''}$	$S_4O_6^{''}$	$SO_4^{''}$	S (element)	
		Concentration (mg. S/l.)				
0	4.4	1030	0	40	0	10^6
42	4.3	1020	10	30	nt	10^4
114	4.3	945	70	80	nt	10^7
138	4.0	120	680	310	tr.	10^7
162	2.2	0	0	1070	tr.	10^4

nt = not tested.

The results given in Table 5 show that during the destruction of thiosulphate, tetrathionate and sulphate were formed, but that there was little change in pH value until thiosulphate had completely disappeared. Tetrathionate was only found during a period of 24 hr. at the fifth to sixth day of incubation. After the complete disappearance of thiosulphate, tetrathionate was converted to sulphate with a sharp drop in pH value.

The effect of concentration of thiosulphate on the course of reaction was studied in the manner described for *Th. concretivorus*. Table 6 records that, as noted by Starkey, the time taken for the pH value to decrease increases with concentration of thiosulphate. Thiosulphate was completely oxidized in media containing concentrations of 0.5 % (w/v) and 1 % (w/v), but with concentra-

Table 6. *Effect of thiosulphate concentration on the oxidation of thiosulphate by Thiobacillus thiooxidans*

Cultures in medium 5, containing various concentrations of sodium thiosulphate were analysed at intervals after inoculation.

Initial thiosulphate concentration (% Na ₂ S ₂ O ₃)	Period of incubation (hr.)	pH value	Form of S				Viable count (organisms/ ml.)
			S ₂ O ₃ ²⁻	S ₄ O ₆ ²⁻ Concentration	SO ₄ ²⁻ mg. S/l.	S (element)	
1	0	4.6	2,580	0	30	0	10 ⁶
	42	4.2	2,455	70	110	nd	10 ⁶
	114	4.2	2,080	220	250	10	10 ⁵
	162	4.3	1,785	280	350	90	10 ⁷
	210	4.3	885	780	710	90	10 ⁸
	306	1.8	0	0	2,430	190	10 ⁷
	618	nd	nd	nd	nd	10	10 ⁸
2	0	4.6	5,250	0	0	0	nd
	144	4.2	4,990	130	90	40	10 ³
	240	4.3	4,965	90	155	nd	nd
	264	4.1	4,870	80	250	50	10 ⁵
	600	4.0	4,790	180	250	80	10 ⁵
4	0	4.9	10,510	0	0	0	nd
	144	4.5	10,485	0	25	10	10 ⁴
	264	4.5	10,075	0	405	nd	10 ⁵
	408	4.5	9,830	0	640	40	10 ⁴
	600	3.9	9,175	70	1,025	310	0

nd = not determined.

tions of 2 % (w/v) and 4 % (w/v), only about one-tenth was oxidized, even after 25 days' incubation. The low viable counts indicate that the higher concentrations of thiosulphate exerted an inhibitory effect. Tetrathionate was found, but the amount produced was less with the higher concentrations of thiosulphate. With a 1.0 % (w/v) thiosulphate the course of oxidation was similar to that described for 0.5 % (w/v); thiosulphate was first oxidized to tetrathionate and sulphate with little change in pH value. In this case, however, tetrathionate was detectable during a much longer period (168 hr.). It reached a maximum concentration when all thiosulphate had been oxidized, and was then converted to sulphate with rapid decrease in pH value. Proportionately much more elementary sulphur was formed from 1 % than from 0.5 % thiosulphate, suggesting that tetrathionate was partly oxidized to acid before the complete disappearance of thiosulphate. The low and diminishing tetrathionate/sulphate ratio during the period of thiosulphate destruction supports this view.

Tetrathionate oxidation. Medium 5, in which 0.5% (w/v) potassium tetrathionate replaced thiosulphate, was repeatedly inoculated, but neither growth nor tetrathionate oxidation could be established. With this organism, like *Th. concretivorus*, it is difficult to understand its inability to metabolize tetrathionate as a primary source of sulphur, while the tetrathionate formed during oxidation of thiosulphate media is rapidly destroyed.

Oxidation of elementary sulphur by *Th. thiooxidans* was studied by Lipman, Waksman & Joffe (1921), Waksman (1922*a, b*), Waksman & Starkey (1923), Starkey (1925). Our re-examination of this oxidation process in medium 6 confirmed the findings of the above authors that sulphur is converted directly to free sulphuric acid without the formation of any intermediate products.

H₂S oxidation. *Th. thiooxidans* was inoculated into medium 5 without thiosulphate, and incubated in air containing H₂S. A viable count was obtained even after 48 days incubation, by which time the pH value had decreased to 2.2. H₂S was oxidized as shown by the increase in total dissolved sulphur from 0 to 472 mg./l.; the main product was sulphuric acid. As with *Th. concretivorus*, a little elementary sulphur accumulated towards the end of the incubation period; this suggests that the oxidation goes through elementary sulphur. The amount of sulphur formed by *Th. thiooxidans* was much less than that formed by *Th. concretivorus*. The ability of *Th. thiooxidans* to oxidize H₂S has been the subject of contradictory statements in the literature, but the present experiments definitely establish its ability to utilize H₂S under the conditions described. Its mode of oxidizing sulphide appears to be identical with that of *Th. concretivorus*.

Thiobacillus X

Thiosulphate oxidation. Oxidation of thiosulphate by two strains was studied. As they oxidized thiosulphate in essentially the same way, results for one strain only (X44) are given in Fig. 1. The final products of oxidation were tetrathionate, sulphate, free sulphuric acid, and very small amounts of free sulphur and pentathionate; the pH value fell from 6.6 to 3.3. The oxidation occurred in three stages. During the first 45 hr. there was a slow increase in sulphate and fall in pH value, but no polythionates could be detected, nor was any free sulphur present. About 10% of the thiosulphate was oxidized during this period and the bacterial count increased from 10⁶ to 10⁸ organisms/ml. In the next 24 hr. the remaining 90% of the thiosulphate was oxidized, the bacterial count increased to 9/ml. and there was little fall in pH value. There was accelerated production of sulphate and rapid production of polythionate (almost entirely tetrathionate). The third stage immediately followed the disappearance of thiosulphate. The pH value decreased from 6.6 to *c.* 3, tetrathionate concentration decreased and the sulphate concentration increased (although more slowly than in the previous period). A thin pellicle, presumably of sulphur, became visible. This third stage occupied 27 hr., during which the bacterial count fell from 10⁹ to 10⁷ organisms/ml. Following the sharp decrease in pH value there was a further slow decrease to an ultimate value of pH 3.0, accompanied by a slow decrease in polythionate and an increase in sulphate

concentration. The bacterial count decreased and the culture became sterile after 120 hr., although a considerable amount of polythionate remained. The inability of this organism to carry tetrathionate oxidation to completion suggests that it is intolerant of pH values less than 3.0. This point was examined by adding a suspension of organisms to sterile saline buffered to different pH values; it was found that on subculture into medium 2, organisms exposed to

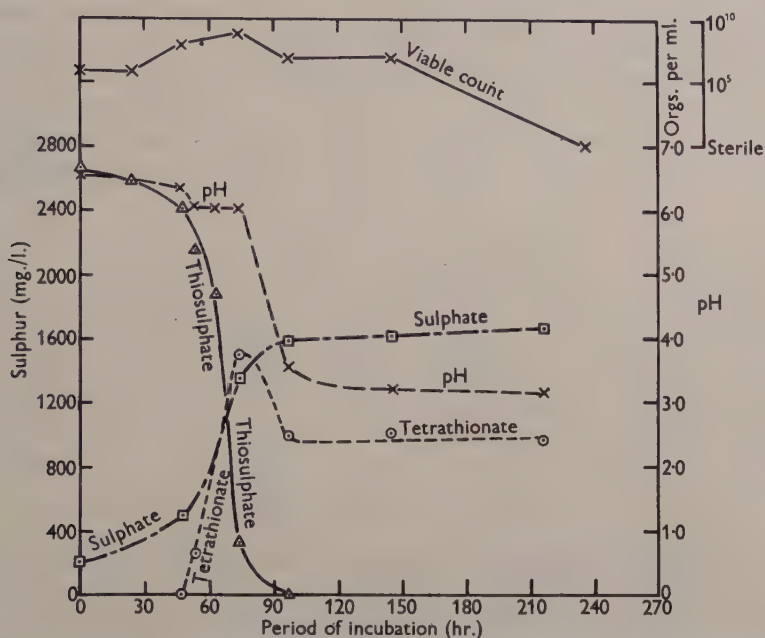


Fig. 1. Oxidation of thiosulphate by *Thiobacillus X* (strain X44). Cultures in medium 2 containing 1% (w/v) $\text{Na}_2\text{S}_2\text{O}_3$ were analysed at intervals after inoculation.

pH values less than 3.0 for 30 min. failed to grow. Further support for this view was obtained by making the usual analyses in a thiosulphate culture in which the pH value, by continuous readjustment, was prevented from becoming less than 4.7 during the whole period of incubation. Under these conditions tetrathionate was completely converted to sulphate, and the rate of increase of sulphate concentration following thiosulphate disappearance was much greater than in the unadjusted culture. Whereas the unadjusted culture was sterile within 5 days of completion of thiosulphate oxidation, a count of 10^7 viable organisms/ml. was found 12.5 days after thiosulphate oxidation had ceased in the adjusted culture.

Tetrathionate oxidation. Tetrathionate was formed as an intermediate in thiosulphate oxidation, and was subsequently converted partially to sulphate and free acid. The strain used was inoculated into medium 2, containing 0.5% (w/v) tetrathionate, instead of thiosulphate, and analysed at intervals as before (Table 7). The strain grew well and oxidized tetrathionate rapidly, the final products being sulphate and some pentathionate; some tetrathionate

remained unoxidized. The oxidation was accompanied by a decrease in pH value from 6.7 to 3.3. Within 30 hr. after inoculation, rapid oxidation of tetrathionate began, and its destruction was associated with rapid increase in sulphate concentration and a sharp decrease in pH value from 6.6 to 3.5. A little thiosulphate appeared between 20 and 45 hr. but later disappeared.

Table 7. *Oxidation of tetrathionate by Thiobacillus X (strain X 44)*

Cultures in medium 2 with potassium tetrathionate instead of thiosulphate were analysed at intervals after inoculation.

		Form of S					
Period of incubation (hr.)	pH value	$S_2O_3^{2-}$	$S_4O_6^{2-}$	$S_5O_6^{2-}$	SO_4^{2-}	S	Viable count (organisms/ml.)
						(element)	
		Concentration mg. S/l.					
20	6.6	5	2100	0	80	0	10^6
45	6.2	55	1810	0	380	tr.	10^6
68	3.9	0	1510	0	590	10	10^6
74	3.8	0	nd	nd	nd	nd	10^7
93	3.6	0	1540	0	600	tr.	10^7
141	3.6	0	1560	0	660	tr.	10^6
237	3.5	0	1050	570	640	tr.	10^3
573	3.3	45	1150	170	850	20	Sterile
Control							
45	6.7	5	2080	0	140	0	Sterile
573	6.5	205	1290	540	200	0	Sterile

tr. = trace; nd = not determined.

When the pH value had decreased to 3.5, oxidation of tetrathionate, sulphate formation, and further fall in pH value all practically ceased. The bacterial count decreased shortly after and eventually the culture became sterile. These results are essentially the same as those described for the third stage of thio-sulphate oxidation by this organism. The appearance of pentathionate after c. 10 days incubation may have been due to a chemical conversion of tetrathionate after bacterial activity had ceased, as the viable count had then fallen to 10^3 organisms/ml. The presence of pentathionate in the sterile control after 24 days incubation indicated that chemical conversion of tetrathionate to pentathionate could occur. These results confirm the ability of this organism to oxidize tetrathionate with the formation of sulphate and acid.

Oxidation of elementary sulphur. The strain was inoculated into medium 2 containing 10 g. sulphur/l. in place of thiosulphate and analysed at intervals. The organism grew with elementary sulphur which was oxidized directly to sulphuric acid as indicated by an increase in sulphate and a decrease in pH value. No immediate products were detected at any stage of the incubation.

The rate of oxidation, indicated by the rate of acid formation, during the period of maximum activity of this organism was only about 10% of that exhibited by *Th. concretivorus* and *Th. thiooxidans*. The oxidation of elementary sulphur by this organism also differed from those organisms, in that the amount of acid formed before the culture became sterile and oxidation ceased was less. Only 0.061% (w/v) SO_4^{2-} was formed by *Thiobacillus X*, compared with values

between 1 and 10 % with the two high acid-formers; the pH value in this case fell only to 3.1, whereas with the other two organisms it became less than 1.0.

H₂S oxidation. Medium 2 lacking any form of oxidizable sulphur was inoculated and incubated in air containing 200 p.p.m. of H₂S. A high viable count was maintained for 24 days; thereafter the count decreased and the culture was sterile after 47 days. Sterility was again associated with decrease in pH value to 3.0, as happened with elementary sulphur and thiosulphate. During the early stage of oxidation only sulphate sulphur increased (owing to the formation of sulphuric acid), but towards the end of the incubation period, when the viable count had decreased, there was also an increase in elementary sulphur. This suggests that the conversion of H₂S to sulphate was through the intermediate formation of elementary sulphur. There was no evidence that thiosulphate or polythionates were formed at any stage.

Thiobacillus thioparus

Thiosulphate oxidation. The strain *Thiobacillus X* was described (Parker, 1947) as *Th. thioparus*-like principally because of (a) its ability to form a pellicle when grown with thiosulphate in liquid culture in a test tube, (b) its very slow oxidation of elementary sulphur and (c) the fact that growth ceased when the pH value fell to 3-4. A detailed examination of an authentic strain of *Th. thioparus* was therefore made for comparison with *Thiobacillus X*. Medium 2, containing 1 % (w/v) sodium thiosulphate, was inoculated from a fresh slope culture, and observations were made as before (Table 8). The

Table 8. *Oxidation of thiosulphate by Thiobacillus thioparus*

Cultures in medium 2 were analysed at intervals after inoculation.

Period of incubation (hr.)	pH value	Form of S			Viable count (organisms/ml.)
		S ₂ O ₃ ²⁻	SO ₄ ²⁻	S (element)	
		Concentration mg. S/l.			
0	6.6	2670	40	0	10 ⁶
87	6.5	2605	110	0	10 ⁷
187	6.4	2355	290	80	10 ⁷
186	6.3	1005	1008	680	10 ⁷
208	6.3	570	1260	870	10 ⁷
257	6.0	0	1630	1090	10 ⁷
329	5.5	0	1660	1040	10 ⁷
521	4.9	0	1720	980	10 ⁶
689	4.7	0	1720	970	10 ⁵

ultimate products of oxidation of thiosulphate were sulphate and elementary sulphur; polythionates were not detected at any stage of incubation. A decrease in pH value from 6.6 to 4.7 indicated that some of the sulphate existed as H₂SO₄. During the oxidation of thiosulphate, sulphate and sulphur were formed in the ratio of 6 to 4, and the pH value decreased from 6.5 to 6.0. This supports the observations of Starkey (1935*a*). After the complete disappearance of thiosulphate, there was a further decrease in pH value to 4.7, accompanied

by a decrease in the amount of elementary sulphur and a corresponding rise in sulphate concentration. This confirmed the suggestion of Starkey (1935*a*) that the decrease in pH value which follows the disappearance of thiosulphate is due to oxidation of sulphur. The continued viability of the culture for at least 18 days after the disappearance of the thiosulphate, when elementary sulphur was the only available sulphur source, suggests that this conversion of elementary sulphur to sulphuric acid is biological. The slow oxidation of elementary sulphur eventually ceased when the pH value dropped to 4.5. This occurs when only a small proportion of the sulphur first formed had been converted into sulphuric acid. This organism did not produce tetrathionate from thiosulphate.

Tetrathionate oxidation. Medium 2 without thiosulphate, but containing 0.5% (w/v) tetrathionate, was inoculated with *Th. thioparus*. After 24 days of incubation less tetrathionate had disappeared from the incubated flasks than from the sterile controls, although a high viable count in the inoculated flask was always found. A decrease in pH value from 6.6 to 5.9 in the inoculated flasks, compared with a stable pH 6.4 in the sterile control, suggested that oxidation of some sulphur compound had occurred. The presence of 205 mg. thiosulphate sulphur/l. in the sterile controls suggests that thiosulphate was formed by chemical decomposition of tetrathionate and then oxidized by the organisms. It seems probable that this organism does not oxidize tetrathionate.

Oxidation of elementary sulphur. Starkey (1934*a*) stated that *Th. thioparus* oxidizes elementary sulphur. Re-examination of the strain used during the present work confirmed that finding. Sulphur was oxidized to sulphuric acid without the formation of detectable intermediate products. The rate of oxidation was similar to that of *Thiobacillus X* and was much less than for *Th. thiooxidans* and *Th. concretivorus*; the amount of sulphur oxidized was also much less (650 mg./l.). With this organism oxidation ceased when the pH value dropped to 4.4, as happened with thiosulphate.

H₂S oxidation. *Th. thioparus* was inoculated into media 1, 2, 3 and 4, containing no oxidizable form of sulphur, and incubated in air containing 200 p.p.m. H₂S. No growth occurred, so this organism cannot oxidize hydrogen sulphide. Starkey did not specifically mention gaseous hydrogen sulphide, but stated that when examined by him the organism did not utilize sulphides. Beijerinck (1904) recorded that both calcium and hydrogen sulphides were utilized by the original culture of *Th. thioparus* isolated by Nathansohn.

Thiobacillus novellus

Thiosulphate oxidation. The organism was examined in the usual way, using medium 2 with 1% (w/v) thiosulphate. The rate of oxidation by this strain used was very much slower than with any of the previous four organisms; less than 20% of the thiosulphate was oxidized in 40 days. The only product of oxidation was sulphate, and its formation was accompanied by a slow decrease in pH value; no tetrathionate was formed. The bacterial count indicated a low degree of bacterial activity. These results confirm Starkey's observations (1935*a*) on this organism.

Tetrathionate oxidation. When medium 2, without thiosulphate, but containing 0.5 % (w/v) potassium tetrathionate, was inoculated with this organism it became sterile within 15 days, indicating inability to survive or grow with tetrathionate.

Oxidation of elementary sulphur. Inoculation into medium 2 with elementary sulphur in place of thiosulphate indicated that, while viable organisms could be recovered 32 days later, no detectable oxidation of sulphur to sulphate took place. This agrees with Starkey's finding (1935*b*) that the organism does not oxidize free sulphur.

H₂S oxidation. As Starkey found, the organism did not oxidize hydrogen sulphide when inoculated into medium 2 or 4 with hydrogen sulphide as the sole source of sulphur.

'M' cultures

Thiosulphate oxidation. Three strains of this group, M.20, M.77 and M.79, were selected for detailed study because they were the best oxidizers of thiosulphate. As the course of the reaction with the three strains was similar the results from one strain only (M.79) are shown in Table 9.

Table 9. *Oxidation of thiosulphate by 'M' organism, strain M.79*

Cultures in medium 2 were analysed at intervals after inoculation.

Period of incubation (hr.)	pH value	Form of S						Total S (by addition)	Viable count (organisms/ml.)
		S ₂ O ₃ ^{''}	S ₄ O ₆ ^{''}	S ₅ O ₆ ^{''}	S ₃ O ₆ ^{''}	SO ₄ ^{''}	S (element)		
		Concentration mg. S/l.							
0	6.6	2640	0	0	0	80	0	2720	nd
22	6.6	2630	0	nd	nd	100	0	nd	10 ⁶
46	6.5	nd	nd	nd	nd	nd	nd	nd	nd
71	6.6	2440	nd	nd	nd	150	nd	nd	10 ⁴
166	7.3	1255	880	470	0	410	10	3025	10 ⁷
238	7.5	1020	1000	330	0	360	20	2730	10 ⁷
384	7.5	815	1680	30	180	nd	20	nd	10 ⁶
502	7.8	535	1230	330	240	420	30	2785	10 ⁴
695	7.6	395	nd	nd	nd	nd	nd	nd	10 ⁶
1367	7.0	365	490	590	460	7.80	75	2755	10 ⁷
1775	6.8	nd	nd	nd	nd	nd	nd	nd	nd

nd = not determined.

Strain M.79 destroyed 87 % of the thiosulphate during 57 days. The products of oxidation after 56 days of incubation were a mixture of tri-, tetra- and pentathionate, some sulphate and a little elementary sulphur. The pH value at first increased from 6.6 to 7.8 and then fell slowly to 7.0. The bacterial count remained high throughout the period of incubation. There was a rapid disappearance of thiosulphate early in the incubation, becoming slower after *c.* 8 days and stopping before all the thiosulphate had disappeared. The period of rapid thiosulphate oxidation was associated with the appearance of a considerable amount of tetrathionate and a sharp increase in pH value; this increase distinguishes these strains from those organisms already described. There was a slight increase in sulphate concentration and some pentathionate formation, but no elementary sulphur was formed in this stage. The subsequent stage of slower thiosulphate oxidation was associated with a slow decrease in

tetrathionate, the formation of tri- and pentathionate, and a slow increase in sulphate. Only minute amounts of elementary sulphur were formed, even after prolonged incubation.

Tetrathionate oxidation. The 'M' strains produced tetrathionate from thiosulphate, but showed little ability to destroy it. They were inoculated into medium 2 in which 0.5 % (w/v) tetrathionate replaced thiosulphate, but could only be recovered in small numbers after 14 days.

Oxidation of elementary sulphur. Strain M.79 was incubated in medium 2 with elementary sulphur in place of thiosulphate. It persisted in the medium for at least 32 days. Although no change occurred in the pH value of the medium, there was a very slight increase (from 49 to 106 mg./l.) in the sulphate content, which suggests that this strain could oxidize sulphur very slowly. The pH value did not decrease, probably because the amount of acid formed was too small to affect the phosphate-buffered medium. The rate of sulphate formation was lower than with *Thiobacillus X* and *Th. thioparus*.

H₂S oxidation. Although these 'M' strains remained viable for periods up to 44 days when inoculated into medium 4 with hydrogen sulphide as source of sulphur, there was no evidence of hydrogen sulphide oxidation.

Thiobacillus B (Waksman) and strains 'T' and 'K' (Trautwein)

Thiosulphate oxidation. *Thiobacillus B* (Waksman) and strains 'T' and 'K' (Trautwein) were examined in medium 2. The results confirm Starkey's observations (1935*a*) that these cultures oxidize thiosulphate primarily with the formation of alkali and tetrathionate, accompanied by an increase in pH value. This was followed later by the appearance of trithionate, pentathionate and some sulphate, with a slow decrease in pH value.

Tetrathionate oxidation. These strains were similarly examined to determine their ability to survive in medium 2 in which 0.5 % (w/v) tetrathionate replaced thiosulphate. All three strains were recovered after 15 days incubation, but since the pH value of the cultures did not differ from that of the control, it seems unlikely that they were capable of oxidation of tetrathionate.

Oxidation of elementary sulphur. These strains were inoculated into medium 2 with elementary sulphur in place of the thiosulphate. Strains 'B' and 'T' survived for 32 days, but strain 'K' was not viable after 7 days. There was no analytical evidence to suggest that any of the strains oxidized sulphur to sulphate, which confirms Starkey's findings (1934*a*). Their behaviour is thus similar to that of *Th. novellus* in this respect.

H₂S oxidation. *Thiobacillus B* and strains 'T' and 'K' were inoculated into medium 4 with hydrogen sulphide as source of sulphur. Cultures of *Thiobacillus B* and strain 'K' became sterile within 24 hr. It was earlier suggested that H₂S in high concentration is toxic to *Th. concretivorus* and the rapid death of the cultures of *Thiobacillus B* and of strain 'K' suggests that H₂S may be toxic to these organisms also. Strain 'T', however, grew. Parker (1947) showed that H₂S in air, passed over an alkaline solution, forms small amounts of thiosulphate and polythionate. It seems probable that a small amount of thiosulphate was formed chemically, in the 'T' culture medium, and that this

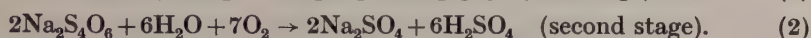
enabled strain 'T' to grow. With *Thiobacillus B* and strain 'K', however, the toxic effect of H_2S would appear to have prevented growth, despite the presence of available thiosulphate.

DISCUSSION

Oxidation of thiosulphate

Thiosulphate is the form of oxidizable sulphur most widely utilized by *Thiobacillus* spp.; the nine strains studied all oxidized thiosulphate readily. The course of oxidation, however, varies with the strain, four different courses being observed.

(1) Thiosulphate is oxidized by *Th. concretivorus* and *Th. thiooxidans* first to tetrathionate and sulphate. In a further stage the tetrathionate is oxidized to sulphate and free sulphuric acid according to equations such as



The production of free acid in the second stage explains the rapid decrease in pH value after the disappearance of thiosulphate.

Waksman & Starkey (1923) stated that *Th. thiooxidans* oxidized thiosulphate directly to sulphate and sulphuric acid; the present examination revealed that there is an intermediate production of tetrathionate. Waksman & Starkey (1923) only made observations at infrequent intervals, and Starkey (1934*b*), when examining this organism for polythionate formation, also only made observations after 7 days incubation, when 7% of the thiosulphate had been oxidized, and after 28 days, when the whole of it had disappeared. In view of the transient occurrence of the tetrathionate during incubation it is not surprising that its formation was overlooked.

Th. concretivorus differs from *Th. thiooxidans* in that the former oxidizes thiosulphate completely from initial concentrations up to 4%, whereas *Th. thiooxidans* is markedly inhibited by 2% and almost completely inhibited by 4%.

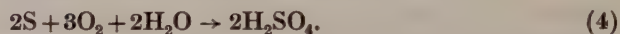
With *Th. concretivorus* in concentrations of 2 and 4% thiosulphate the decrease in pH value and the occurrence of the maximum tetrathionate concentration before all the thiosulphate has disappeared suggests that towards the end of the thiosulphate oxidation, thiosulphate and tetrathionate oxidation are taking place simultaneously. With the 2 and 4% concentrations, the amount of elementary sulphur formed is proportionately much greater than with the lower concentrations. The formation of 100 mg. S/l. after 26 days from the 4% concentration before complete disappearance of thiosulphate is also consistent with the simultaneous oxidation of thiosulphate and tetrathionate; the acid formed from tetrathionate oxidation could react with unchanged thiosulphate to precipitate sulphur. As a final stage the sulphur so formed may be oxidized to sulphuric acid as, with the 1.0 and 2% initial concentrations of thiosulphate, the concentration of sulphur at the end of the incubation period further decreased.

Oxidation by *Th. thiooxidans* in 2% thiosulphate, although less in extent,

is similar in nature to that by *Th. concretivorus* at concentrations of 2 and 4%. Over the whole period of observation tetrathionate and elementary sulphur could be detected; the sulphate showed a slow increase and the pH value a slow decrease. Although only a little thiosulphate was oxidized it would appear that thiosulphate and tetrathionate oxidation occurred simultaneously.

Thiosulphate oxidation by *Thiobacillus X* appears also to occur in two stages according to equations such as (1) and (2) above. With this organism, however, the second stage does not go to completion; the culture becomes sterile at a pH value of 3.0, although some tetrathionate remains unoxidized. The increase in sulphate without any tetrathionate accumulation at the beginning of the incubation, and the fact that the pH value does not increase during the rapid oxidation of thiosulphate in the second stage, may be because the two reactions occur simultaneously from the beginning of the incubation, although the formation of the tetrathionate oxidase would appear to be slower than that of the thiosulphate oxidase.

(2) Thiosulphate is oxidized by *Th. thioparus* to sulphate and elementary sulphur, followed by a slight and slow oxidation of the sulphur to sulphuric acid, with a decrease in pH value to 4.3, according to equations such as (3) and (4):



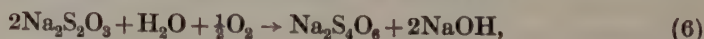
As with *Thiobacillus X* the extent of acid formation appears to be limited by acid tolerance of the organisms; this was confirmed by examination of the pH tolerance of the organism in buffered saline. The first stage of this oxidation by *Th. thioparus* was described by Starkey (1935*a*); it was confirmed in the present work. Starkey also suggested that the decrease in pH value was probably due to oxidation of the sulphur first formed; this suggestion has been confirmed by the present work.

(3) Thiosulphate may be converted by *Th. novellus* directly to sulphate and sulphuric acid, with decrease in pH value according to an equation such as (5):



As with *Thiobacillus X* and *Th. thioparus*, the amount of acid formed appears to be limited by the acid tolerance of the organism; this was confirmed by separate examination in buffered saline. The present observations confirm those of Starkey (1935*a*) with regard to the route of thiosulphate oxidation by *Th. novellus*. It had been considered that the thiosulphate oxidation by *Th. novellus* was identical with that produced by *Th. thiooxidans*, but the present observations concerning the intermediate production of tetrathionate by *Th. thiooxidans* indicates a marked difference between the two organisms.

(4) Thiosulphate can also be converted to sulphate and tetrathionate, with an increase in pH value, according to an equation such as (6):



followed by a further small conversion of tetrathionate to tri- and pentathionate, and a decrease in pH value, sometimes back to the initial value at

inoculation. Oxidation in this way by the 'T' and 'K' strains of Trautwein and by *Thiobacillus B* was described by Starkey (1935a); the present results are in agreement with those observations. Starkey suggested that the conversion of tetra- to tri- and pentathionate was a purely chemical change; the findings of high viable counts throughout the incubation period in the present work suggests that the organisms may be involved. However, the detection of pentathionate in sterile controls of medium 2 containing tetrathionate instead of thiosulphate, indicates that pentathionate may arise from tetrathionate by chemical change. The facultative autotrophic 'M' strains isolated from concrete, attack thiosulphate in the same way as the 'B', 'T' and 'K' strains. The products of oxidation are identical, but the cultures vary in their rate of oxidation.

Oxidation of tetrathionate

Tetrathionate as a sole source of oxidizable sulphur is oxidized only by *Thiobacillus X*; the products of oxidation are sulphate and sulphuric acid according to equation (2). Oxidation of tetrathionate is incomplete because of the acid intolerance of the organism. In view of the intermediate formation of tetrathionate from thiosulphate by *Th. thiooxidans*, *Th. concretivorus*, the 'M', 'B', 'T' and 'K' strains as well as by *Thiobacillus X*, it is of interest that only *Thiobacillus X* can oxidize tetrathionate when it is supplied as the sole sulphur source.

Oxidation of elementary sulphur

Elementary sulphur is oxidized direct to sulphuric acid; this is brought about only by the four strictly autotrophic strains *Th. thiooxidans*, *Th. concretivorus*, *Thiobacillus X* and *Th. thioparus*. The rate of acid formation and ultimate concentration of acid formed differs among the strains, and the latter is dependent on the acid tolerance of the strain. *Th. thiooxidans* and *Th. concretivorus* give the highest rates and the highest final concentration of acid, *Thiobacillus X* shows a slower rate and lower final concentration, while the rate of sulphur oxidation by *Th. thioparus* and the final acid concentration is even less.

Oxidation of H₂S

Oxidation of gaseous H₂S in air passed over appropriate liquid cultures is only brought about by *Th. thiooxidans*, *Th. concretivorus* and *Thiobacillus X*. The only product of oxidation of H₂S is sulphuric acid, although the accumulation of small amounts of elementary sulphur towards the end of incubation in acid media, and of thiosulphate in alkaline media, suggests that the formation of sulphur and thiosulphate by purely chemical means occurs first, this sulphur or thiosulphate then being oxidized to sulphuric acid by the organism. Oxidation of H₂S by *Th. thiooxidans* and *Th. concretivorus* produces only enough acid to give a pH value of 2.0 under the given conditions, while oxidation of elementary sulphur and thiosulphate by the same organisms produces an acidity equivalent to a pH value of less than 1.0. As the same organisms are involved, difference in acid tolerance cannot be responsible. If elementary sulphur formed chemically from H₂S be an essential preliminary product in

acid media, it is possible that this reaction does not proceed below pH 2.0, and the activity of the organisms ceases at this pH because no more sulphur becomes available. Some support for this hypothesis is derived from other investigations in these laboratories which indicate that H_2S dissolved in water is chemically oxidized by dissolved oxygen at very much slower rates under acid conditions than under alkaline conditions. It is, of course, also possible that the pH range of the enzyme systems involved in H_2S oxidation is different from those involved in the oxidation of elementary sulphur and thiosulphate. In contrast to the behaviour of *Th. concretivorus* and *Th. thiooxidans* in H_2S the pH value of the medium decreases to 3.0 with *Thiobacillus X*; this value is the limit of its acid tolerance with elementary sulphur or thiosulphate as sulphur source.

Identity of organisms isolated from concrete

Th. concretivorus. This organism oxidizes thiosulphate, elementary sulphur and hydrogen sulphide according to the same reactions as *Th. thiooxidans*, and like *Th. thiooxidans* fails to utilize tetrathionate. It differs from *Th. thiooxidans* in being inhibited by higher concentrations of thiosulphate. The two organisms are closely related with regard to oxidation of sulphur compounds, but, as described earlier (Parker, 1945), they differ in their nitrogen requirements. *Th. thiooxidans* utilizes ammonia but is inhibited by nitrate, whereas *Th. concretivorus* utilizes ammonia or nitrate.

Thiobacillus X. This organism was previously considered to be closely related to *Th. thioparus*, but the present study reveals that the two organisms exhibit marked differences with regard to their oxidation of sulphur compounds. *Th. thioparus* oxidizes thiosulphate with formation of elementary sulphur and sulphate, whereas *Thiobacillus X* converts thiosulphate first to sulphate and tetrathionate followed by a partial destruction of tetrathionate, forming sulphate and free sulphuric acid. *Th. thioparus* does not oxidize tetrathionate or H_2S , whereas *Thiobacillus X* readily oxidizes both compounds. The only point of similarity is their common power to oxidize elementary sulphur slowly to sulphuric acid. *Thiobacillus X* must therefore be considered a different species from *Th. thioparus*, and with regard to oxidation of sulphur compounds is much more closely related to *Th. thiooxidans* and *Th. concretivorus*. All three organisms utilize thiosulphate, elementary sulphur or H_2S , and the route of oxidation of thiosulphate is by the same two reactions in each case. *Thiobacillus X* differs from *Th. thiooxidans* and *Th. concretivorus*, however, in its acid tolerance. Oxidation of thiosulphate ceases with *Thiobacillus X* at pH 3.0, leaving sulphate, free sulphuric acid and some unchanged tetrathionate as the products of oxidation, while oxidation by *Th. thiooxidans* and *Th. concretivorus* only ceases at a pH value less than 1.0, the only products of oxidation being sulphate and free sulphuric acid. *Thiobacillus X* must therefore be considered as distinct from *Th. thioparus* on the one hand and *Th. thiooxidans* and *Th. concretivorus* on the other. Before classing *Thiobacillus X* as a new species it is desirable to review the published literature on *Th. thioparus*. The strain of *Th. thioparus* examined in these studies was one isolated by Starkey in 1935, and Starkey (1935*a*) considered it identical with the organism

isolated by Nathansohn (1902) and which was re-examined and named by Beijerinck (1904).

The original organism of Nathansohn was said to produce tetrathionate by oxidation of thiosulphate but Starkey was unable to detect tetrathionate as a product of thiosulphate oxidation by his strain, and suggested that the original Nathansohn strain was contaminated with an organism similar to the 'B', 'T' and 'K' strains. Production of acid was not specifically mentioned by Nathansohn or Beijerinck, but slight acid formation by the original culture cannot be excluded.

Nathansohn's original strain was said to produce sulphur from thiosulphate, and Starkey's strain produces sulphur and sulphate in the ratio of 4:6. *Thiobacillus X* produces a thin pellicle of sulphur, but very much less than that produced by Starkey's organism. It is possible that the sulphur formed by Nathansohn's original organism and by *Thiobacillus X* is due to chemical reaction between thiosulphate and tetrathionate, whereas the sulphur formed by Starkey's strain is directly produced from thiosulphate.

According to Beijerinck (1904), *Th. thioparus* can oxidize thiosulphate, tetrathionate, sulphur, sulphides and H_2S . In view of the production of tetrathionate from thiosulphate by *Thiobacillus X* and its ability to oxidize thiosulphate, tetrathionate, sulphur and H_2S , it seems more likely that the original strain of Nathansohn was not impure; that its oxidizing powers as described by that author were correct; and that the strain *Thiobacillus X* isolated from concrete is identical with or very similar to the original *Th. thioparus*. Starkey's strain, which he considered identical with the original *Th. thioparus*, would appear to differ from both *Th. thioparus* as isolated by Nathansohn and *Thiobacillus X*.

Since the completion of the present work, Vishniac (1952) published a manometric study of the oxidation of thiosulphate by a strain considered identical with Starkey's strain of *Th. thioparus*. It was found that this organism gave qualitative tests for tetrathionate, and that its oxygen uptakes were consistent with tetrathionate formation. Vishniac considered that oxidation of thiosulphate by the strain used proceeded through tetrathionate, trithionate and dithionate to sulphate. It appears likely that Vishniac's strain was very similar to *Thiobacillus X* and to *Th. thioparus* as originally described by Nathansohn; although Vishniac stated that a culture of Starkey's strain of *Th. thioparus* appeared identical with his own, if examined closely it seems probable that they would differ in the same way as *Thiobacillus X* and *Th. thioparus* differed in the present work.

'M' strains. Those cultured from concrete appear to be closely related to the 'T' and 'K' strains of Trautwein and *Thiobacillus B* with regard to oxidation of sulphur compounds. All strains oxidize thiosulphate with the formation of tetrathionate, and with an increase in pH value they are similar in their action on the other forms of sulphur examined.

The authors wish to thank Mr J. A. McIntosh, Acting Chief Engineer of Sewerage, Melbourne, and Metropolitan Board of Works, for permission to publish this paper, and Miss E. M. Jackson who assisted with the preparation of media.

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(Received 8 August 1952)

BENTLEY, M. L. (1953). *J. gen. Microbiol.* 8, 365-377.

Enzymes of Pathogenic Fungi

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SUMMARY: Submerged growth of four dermatophytes, *Microsporum canis*, *M. gypseum*, *Trichophyton rubrum* and *T. mentagrophytes* has been obtained by shaking during growth. The respiratory rate ($\mu\text{l. O}_2$ consumed/hr./mg. dry weight) of washed cells from 4-6-day cultures was between 7 and 12. As commonly observed with other fungi, addition of substrate to washed respiring cells did not much increase the respiratory rate. Acetone powders were prepared from cells grown in shaken culture. Such preparations oxidatively deaminated amino-acids slightly, about as much as similar preparations of *Penicillium chrysogenum*. A preparation of the enzyme asparaginase was extracted from *Microsporum gypseum* and some of its characteristics studied.

The metabolic processes of the dermatophytes have only recently received much attention. Vitamin requirements have been demonstrated, for example, for *Trichophyton faviforme* (Georg, 1950), *T. discoides* (Robbins, Mackinnon & Ma, 1942) and *T. album* (Schopfer & Blumer, 1943) and the amino-acid requirements of *T. mentagrophytes* were investigated by Robbins & Ma (1945). Nickerson & Chadwick (1946) studied respiration and assimilation in several species of dermatophytes and the effect of various compounds upon them. Melton (1951) extended this work to shaken cultures of *Microsporum canis*, and Stahl, McQue, Mandels & Siu (1949) studied the sulphur metabolism of *M. gypseum*.

The enzyme systems of these organisms have not been well characterized, although as early as 1895, Macfadyen (1895-6) found an extracellular proteolytic enzyme in the culture fluid of *T. tonsurans*. Bodin & Lenormand (1901) found various extracellular enzymes in cultures of *M. equinum*: one that clotted milk, another which dissolved the clot, and enzymes which liquefied gelatin, hydrolysed egg albumin and coagulated serum. Tate (1929) reported a survey of the distribution of several respiratory, proteolytic and lipolytic enzymes, carbohydrases and urease in acetone powders of species that were representative of the chief groups of dermatophytes.

It has been observed frequently that cultures of the dermatophytes become alkaline during growth, the change mainly being due to the production of ammonia (Nickerson, 1947). These observations suggested that enzymic mechanisms for ammonia production already known in other fungi, e.g. oxidative deamination of amino-acids by L- and D-amino-acid oxidases (Knight, 1948; Horowitz, 1944) and amidases such as urease, asparaginase (Shibata, 1904; Lang, 1904), may operate in the dermatophytes. It also seemed possible

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that specific amino-acid dehydrogenases, deaminases or amino-acid decarboxylases (Gale, 1946) present in bacterial and animal tissues might be found in the dermatophytes.

This paper reports an attempt to investigate enzyme systems responsible for ammonia formation in *T. mentagrophytes*, *T. rubrum*, *M. canis* and *M. gypseum*. Preliminary experiments to detect amino-acid decarboxylases were all negative. Oxidative deamination of amino-acids was observed, but the activity of the systems was low. Attention was next turned to asparaginase; and since this enzyme proved somewhat easier to work with, it was characterized further.

METHODS

Maintenance and cultivation of the dermatophytes. The cultures used in these experiments were obtained from the M.R.C. Medical Mycology Unit at the London School of Hygiene and Tropical Medicine. They were *M. gypseum*, *M. canis*, *T. mentagrophytes* and *T. rubrum* and had been isolated in 1950. Subcultures were maintained on malt agar slopes except for a short time at the beginning of the work when beer-wort slopes were used. Stock cultures were transferred about every 6 weeks. For experimental purposes a series of several tubes was inoculated from stock cultures and incubated for 2 weeks at 22°. Succeeding series were inoculated from a 2-week culture of the preceding series. Under these conditions of rapid transfer, *M. gypseum*, and particularly *M. canis*, tended to form rapidly growing non-sporing mycelia instead of the desired sporulating forms. Thereupon the organisms were transferred to barley grains (Langeron & Milochévitch, 1930); growth then reverted to the sporulating form after 6–8 weeks' incubation at 22°. Upon subsequent subculture on malt agar *M. gypseum* was kept sporulating during about 6 months. *M. canis*, on the other hand, very quickly became non-sporing again on malt agar. Eventually the use of the latter organism was abandoned.

The medium used for experimental work contained glucose (anhydrous; 2%, w/v), mycological peptone (Oxoid; 2%, w/v) and bacteriological yeast extract (Difco; 1%, w/v), made up with distilled water and autoclaved at 15 lb./sq.in. for 15 min. before use. For the preliminary studies on the oxidation and deamination of amino-acids, 100 ml. lots of this medium were placed in 500 ml. Erlenmeyer flasks loosely plugged with cotton-wool. In later experiments in which asparaginase was studied, 75 ml. volumes were used in 250 ml. Erlenmeyer flasks. To inoculate the medium, sterile water or saline (5 ml.) was added aseptically to a 2-week sporulating subculture of the organism. The mycelium and spores were scraped off the agar and, after shaking, the suspension was poured into the culture flask, leaving the agar behind. Initially the washings from two agar slopes were used to inoculate each flask; but it was later found advisable to use four slopes/flask in order to obtain a more uniformly sized mycelial pellet. This procedure did not give a standard inoculum, since the mycelium on the agar slope was not readily wetted and the spores tended to form a film on the sides of the tubes, thus resulting in loss on transfer.

After inoculation the flasks were placed on a shaker which moved at

95–100 cyc./min. with a 9 cm. stroke. Under these conditions the fungi grew in balls or pellets of mycelium throughout the culture fluid. After 4–6 days at c. 18°, growth was sufficient, and a few pellets were then removed aseptically from the medium and cultivated on malt agar slopes to test for contamination. Although a few pellets did not represent the entire culture, this method would have detected gross contamination. The few experiments in which contamination was detected were discarded.

Enzyme preparations. The balls of mycelium were separated from the culture medium by filtration through muslin and were then washed with successive portions of distilled water (total about 2 l.) until the washings were colourless. In early experiments the balls of mycelium were used without further treatment; but for the study of asparaginase they were washed with running tap water for 90–180 min. This procedure, suggested by Dr S. G. Knight, had been found helpful in lowering the endogenous respiration of mycelium of certain *Penicillium* spp.

Acetone powders were prepared from washed mycelium by squeezing out as much water as possible, transferring the mycelial balls to acetone cooled to –10° (no lower) and stirring for 6 min. The mycelial balls were then rapidly filtered-off on a large Buchner funnel, washed twice with acetone at 5° and then dried by sucking air through them. When the cake became whitish buff it was crumbled on to filter-paper and allowed to stand until the acetone vapour had disappeared. The powder was stored at 3° over P₂O₅ in an evacuated desiccator. The yield of acetone powder from six flasks of culture medium was about 1.5 g. In a few experiments dried mycelium was prepared by washing the mycelial balls with running tap water, filtering-off and sucking as dry as possible on a Buchner funnel, and then drying *in vacuo* over P₂O₅ for 18 hr. at room temperature; this material was then stored at 3°.

Chemical determinations. All chemical determinations were carried out on trichloroacetic acid (final concentration, 5 %) filtrates of the reaction mixtures. Free ammonia was distilled-off by a modification of the method of Pucher, Vickery & Leavenworth (1935). Saturated potassium carbonate was added to neutralize the acid of the filtrates and liberate ammonia, which was estimated in the distillate by nesslerization. Aspartic acid and asparagine were determined enzymatically by the method of Krebs (1950); for asparagine determinations asparaginase prepared from guinea-pig serum by ethanol precipitation was added to the system. Asparagine was also determined by estimation of ammonia liberated after 3 hr. hydrolysis in N-hydrochloric acid at 100°. Protein was determined colorimetrically with the phenol reagent of Folin & Ciocalteu using tyrosine as a standard (Minot & Keller, 1936). Oxygen uptake was measured manometrically with a Barcroft apparatus or with a Warburg apparatus; CO₂ was determined with the latter equipment.

RESULTS

An immediate difficulty in studying the four dermatophytes was their high respiration in the absence of added substrate (Table 1). The Q_{O₂} (μl. O₂ consumed/mg. dry wt./hr.) of the mycelial balls alone was usually c. 10–12,

never less than 5. Addition of glucose, amino-acids or organic acids did not produce a much higher value. In some cases the Q_{O_2} in the presence of a substrate was lower than the value in its absence, but such results were frequently not reproducible.

Table 1. *Respiratory quotients of certain dermatophytes in the absence and presence of substrate*

Phosphate buffer and substrate were both 0.016 M; volume 3 ml.; gas-phase air; at 38°.				
Organism	Treatment	Substrate	pH	Q_{O_2}
<i>T. rubrum</i>	Washed	—	7	10.7
		glucose	7	11.3
<i>M. gypseum</i>	Aerated for 3 hr. at c. 18°, then placed for 24 hr. at 3°	—	7	7.6
		glucose	7	12.3
		glucose and glycine	7	8.7
		glucose and succinate	7	10.1
<i>M. canis</i>	As for <i>M. gypseum</i>	—	8	6.8
		L-glutamic acid	8	4.6

Aeration of the mycelial balls for a few hours in phosphate buffer (pH 7) or distilled water, at room temperature, either lowered the respiratory rate in the presence and absence of substrate alike or, when prolonged, abolished activity. Similar aeration followed by storage overnight in the refrigerator did not lower the respiration of the mycelium in buffer alone more than that with substrate. Melton (1951) was able to lower the endogenous respiration of *M. canis* grown in shaken culture by starving the organisms for a day or more. He probably succeeded with the technique because his organisms were grown for 2–3 weeks.

The values for respiratory rate of the mycelial balls shown in Table 1 are considerably higher than those reported by Nickerson & Chadwick (1946), who found values from 0.2 to 2.0 for the Q_{O_2} of *T. rubrum* and *T. gypseum*, but are similar to those reported by Wolf (1948) for shaken cultures of *Penicillium chrysogenum* Q176. The differences are probably due in the main to the fact that Nickerson & Chadwick used 2–4-week surface cultures. Darby & Goddard (1950) showed that the respiratory rate of shaken cultures of *Myrothecium verrucaria* was four times greater than that of static cultures. Differences in age of culture also cause considerable variation in respiratory rate as Wolf (1947) showed with cultures of *P. notatum*.

Acetone powders

To avoid the difficulty of pronounced respiration of the mycelial balls without added substrate, acetone powders of the mycelium from shaken cultures were made. A rough Q_{O_2} computed from results with *M. canis* as μ l. O_2 consumed/hr./mg. acetone powder was compared with that of intact mycelium. Values of this Q_{O_2} = 0.1–0.2 were found for the respiration of the powder in buffer (see Table 2), suggesting damage to the respiratory systems during the acetone treatment. However, this residual respiration, though slight, continued for several hours at a gradually diminishing rate. By addition of yeast

extract or of substrate (glucose or amino-acids) the respiratory rate was doubled. Addition of yeast extract and substrate together produced a Q_{O_2} of 1.0. However, the activity was so low that the significance of these results is not certain.

Table 2. *Oxygen uptake of acetone powders of Microsporum canis with and without substrate*

Experiments (1) and (2): 0.02 M-phosphate, 20 mg. acetone powder.
Experiment (3): 0.03 M-acetate, 40 mg. acetone powder in each vessel.
Concentration of substrates given in brackets. Yeast extract: 0.33 %;
volume 3 ml.; gas-phase, air; at 38°.

Exp. no.	pH	Substrate	Yeast extract	Q_{O_2}
1	6	None	—	0.16
	6	None	+	0.43
	6	Glucose (0.016 M)	+	0.96
2	8	L-glutamic acid (0.033 M)	—	0.55
	8	L-glutamic acid (0.033 M)	+	1.08
3	5	None	—	0.16
	5	Glucose (0.008 M)	—	0.27
	5	L-aspartic acid (0.005 M)	—	0.37
	5	Succinic acid (0.008 M)	—	0.20
	5	L-leucine (0.016 M)	—	0.33

Ammonia formation accompanied the oxygen uptake by the acetone powder respiring in buffer alone, about 0.8–1.5 $\mu\text{mole NH}_3/\mu\text{mole O}_2$ consumed. In the presence of each of several amino-acids increased ammonia formation was observed, which suggested oxidative deamination of the added amino-acid. Oxidative deamination of amino-acids by L-amino-acid oxidase of animal tissues and bacteria has been shown to proceed as indicated in equation (1):



In the presence of catalase, which Tate (1929) showed to be present in all the dermatophytes he tested, the hydrogen peroxide formed in the oxidation is decomposed (equation (2)), so that the overall reaction observed is



One μatom oxygen is consumed/ μmole ammonia formed. Fairly good correspondence with these quantitative relations was observed with aspartic acid as substrate for *M. gypseum*. With the other amino-acids, the activity was so low that agreement with the equations seemed fortuitous (see Table 3). In view of the low O_2 uptake and possible variations of catalase content of the acetone powders, deamination was subsequently followed by direct estimation of ammonia which seemed more sensitive and more reliable than measurement of O_2 uptake.

An L-amino-acid oxidase has been described in acetone powders of *P. chrysogenum* NRRL-1951-B25 and Q176 (Knight, 1948), and in Table 4 these results are compared with those obtained with acetone powders of *M. canis* and *M. gypseum*. Knight established that for L-alanine and L-methionine

1 μ mole ammonia was liberated by the two strains of *Penicillium* for each μ atom O_2 consumed. Presumably the relation held for the other amino-acids tested. Therefore, in Table 4, his results, originally in terms of mm.³ oxygen consumed/50 mg. acetone powder/80 min., have been expressed as μ mole ammonia/50 mg. acetone powder/80 min. The stimulation of ammonia formation by amino-acids observed in the present study of the dermatophytes has

Table 3. *Oxygen uptake and ammonia formation by acetone powders of Microsporium gypseum*

Amino-acids and phosphate buffer (pH 8) were both 0.016 M. 50 mg. acetone powder/vessel. The vessels were incubated $\frac{3}{4}$ hr. at 38° in air.

Addition	Ammonia formed		Oxygen consumed	
	Not corrected (μ mole)	Corrected for blank (μ mole)	Not corrected (μ atom)	Corrected for blank (μ atom)
None	3.94*	—	2.16	—
L-leucine	4.59	0.65	2.85	0.64
L-aspartic acid	9.50	5.56	6.92	4.76
L-phenylalanine	4.75	0.81	2.64	0.48
L-glutamic acid	7.59	3.65	2.78	0.62
Glycine	5.43	1.49	—	—

* The zero time value was 2.93 μ mole ammonia: therefore, 1.01 μ mole were formed during the incubation.

Table 4. *Comparison of activity in deaminating amino-acids of certain dermatophytes and Penicillium chrysogenum NRRL-1951-B-25.*

The experiments with the dermatophytes were done in 0.02 M-phosphate (pH 8). With *Microsporium gypseum*, values designated *a*. 40 mg. acetone powder/tube; remaining tubes, 50 mg. acetone powder/tube. The amino-acids were 0.03 M. Tubes were shaken in air at c. 18°. With *M. canis* 40 mg. acetone powder/tube and amino-acids as follows: DL-asparagine, L-leucine and L-alanine, 0.03 M; L-glutamic acid, L-aspartic acid and L-lysine, 0.01 M. The tubes were incubated 5 hr. in air at 25°.

Organism	Amino-acid	μ mole NH_3 /50 mg. acetone powder/80 min.*	
		Dermatophytes	<i>P. chrysogenum</i> (Knight, 1948)
<i>M. gypseum</i>	L-leucine	0.29 (16 %)	0.37
	L-leucine	0.36 (12 %) <i>a</i>	—
	L-aspartic acid	2.47 (116 %) <i>a</i>	0.62
	DL-aspartic acid	0.75 (26 %) <i>a</i>	—
	L-phenylalanine	0.36 (24 %) <i>a</i>	0.18
	L-glutamic acid	1.69 (94 %) <i>a</i>	0.94
	L-glutamic acid	0.0 <i>a</i>	—
	Glycine	0.66 (28 %) <i>a</i>	0.22
	L-alanine	(-) 0.60 (-20 %) <i>c</i>	5.1
<i>M. canis</i>	DL-asparagine	0.43 (14 %) <i>a</i>	—
	DL-asparagine	1.98	—
	L-glutamic acid	0.55 (23 %) <i>a</i>	0.94
	L-leucine	1.48 (62 %) <i>a</i>	0.37
	L-alanine	(-) 0.48 (-20 %) <i>c</i>	5.1
	L-aspartic acid	1.19 (50 %) <i>a</i>	0.62
	L-lysine	0.0	—

* All values corrected for the blank.

been converted to the same units. Both sets of experiments were carried out at pH 8 in 0.02 or 0.016 M-phosphate buffer (see Table 4). Knight's experiments were carried out at 30°.

L-Alanine was most rapidly attacked by the penicillium preparations and L-aspartic acid by the two dermatophyte preparations; but with the latter the rate was about half that of the former. The activity of the penicillium and *M. canis* and *M. gypseum* toward the other amino-acids was slight and of the same order of magnitude. To give a clearer idea of the quantitative relationship between ammonia formation by the blank (acetone powder and buffer alone) and in the presence of amino-acids, the original increment over the blank expressed as percentage of the blank has been placed in parentheses beside the values in column 3 of Table 4.

The smaller amount of ammonia formed in the presence of L-alanine relative to the blank has been observed several times with *M. canis* and *M. gypseum*. Without further investigation its significance cannot be assessed. However, Schade & Thimann (1940) found much the same in their study of the metabolism of the water mould *Leptomitius lacteus*. Leucine was vigorously oxidized by the mould, but the ratio of ammonia produced to O₂ taken up was lower than that found when the mould respired in buffer alone, indicating that some ammonia was absorbed. Moreover, the R.Q. was lower than that required for complete oxidation of leucine. They explained the observation by assuming that leucine was oxidized to a compound with the formula of alanine and that this product was assimilated.

Dialysed aqueous extracts of acetone powders of *T. mentagrophytes*, *M. gypseum* and *M. canis* deaminated L-aspartic acid; the activity of these extracts toward other amino-acids was very low or negligible. A typical experiment showing the activity of aqueous extracts in deaminating amino-acids is shown in Table 5. Seven ml. of water were added to 500 mg. acetone powder of *M. gypseum*; after stirring for 15 min. and centrifuging, the supernatant was dialysed for 80 min. against running water. One ml. of the dialysed solution was used in each of the five tubes indicated in Table 5. To the residue left after centrifugation, 6 ml. of water were added, and 1 ml. of this suspension was then used in each of the four tubes shown in Table 5. Under these conditions of extraction the activity for deaminating L-leucine and DL-asparagine remained in the residue and only activity toward DL-aspartic acid was extracted, although not completely. Similar treatment of acetone powder of *T. mentagrophytes* also extracted the activity toward aspartic acid and probably that toward phenylalanine.

The evidence thus suggests oxidative deamination of the amino-acids by enzymes in *M. canis*, *M. gypseum* and *T. mentagrophytes*, such as an L-amino-acid oxidase or specific amino-acid deaminases. In this connexion, a study of the metabolism of *Blastomyces dermatitidis* is of interest (Bernheim, 1942). Bernheim found that in the presence of amino-acids, more ammonia was produced than in the control; but, although the oxygen uptakes caused by the amino-acids were great enough to account for their complete oxidative deamination, in no case was the theoretical amount of ammonia recovered.

Furthermore, analyses showed that in the case of L-phenylalanine, no α -amino-nitrogen disappeared, and in the case of D-alanine more α -amino-nitrogen was found at the end of the experiment than had been added as D-alanine.

Table 5. *Activity of extracts of acetone powders of certain dermatophytes in deaminating amino-acids*

Microsporum gypsum. One ml. dialysed extract of acetone powder or 1 ml. of aqueous suspension of the residue after extraction (see text) was incubated in 0.02 M-phosphate buffer (pH 8) with 0.002 M-MgCl₂ and as indicated, 0.016 M-DL-asparagine, 0.03 M-L-leucine or 0.03 M-DL-aspartic acid. Marmite (0.16 %) was added to all the tubes containing the extract (except to the first control) and to all tubes containing suspended residue. Total vol. 3.6 ml. The tubes were incubated in air for 300 min. at c. 18°.

EXTRACT	
Substrate	Ammonia-N evolved in 5 hr. (μ g.)
None	50
None	120
L-asparagine	121
L-leucine	125
DL-aspartic acid	182

SUSPENDED RESIDUE	
Substrate	Ammonia-N evolved in 5 hr. (μ g.)
DL-asparagine	146
L-leucine	153
DL-aspartic acid	180
None	121

Trichophyton mentagrophytes. One ml. of dialysed extract of acetone powder was placed in each tube. The amino-acids were present in a final concentration of 0.03 M and phosphate buffer (pH 8), 0.02 M. The tubes were shaken in air for 214 min. at 22°.

EXTRACT	
Substrate	Ammonia-N evolved in 214 min. (μ g.)
None	57.5
L-glutamic acid	61.7
L-leucine	65.5
DL-aspartic acid	106.0
Glycine	62.7
DL-phenylalanine	80.0
DL-proline	57.5

Asparaginase

Ammonia was produced when acetone powders of *M. canis* or *M. gypsum* were incubated with asparagine (Table 1), suggesting the presence of an asparaginase. So little ammonia was formed when acetone powders of *T. mentagrophytes* and asparagine were incubated together that it was doubtful whether an asparaginase was present in them.

The yield of 1 mole NH₃ and 1 mole L-aspartic acid from 1 mole L-asparagine was established by chemical analyses for ammonia and asparagine and the enzymic analysis for L-aspartic acid and L-asparagine (Krebs, 1950) (Table 6). By virtue of the specificity of the enzymic method for L-aspartic acid, the

recovery of an equivalent of aspartic acid for each mole of L-asparagine disappearing indicated that the natural isomer of aspartic acid was the product of the above reaction. It is also clear from the analytical results that the α -amino nitrogen of asparagine was not attacked. The results of an experiment (no. 3) with acetone powder suspended in phosphate buffer at pH 8 present difficulties. Under these conditions, as shown previously, aspartic acid added to acetone powder stimulated ammonia formation, presumably by deamination of aspartic acid. The most likely reason why the aspartic acid formed upon hydrolysis of asparagine is not deaminated is that the aspartic acid concentration is very far below optimal for its deamination.

Table 6. *Chemical balance of hydrolysis of L- β -asparagine by asparaginase in Microsporum gypseum*

Exp. 1. Veronal extract of acetone powder was dialysed for 1 hr. against running tap water. Experimental tubes contained 1 ml. of dialysed veronal extract, 0.5 ml. of 0.1 M-L- β -asparagine made up in phosphate buffer (pH 8); 1 ml. of 0.067 M-phosphate buffer (pH 8) and water to 3 ml. A duplicate mixture was made and immediately precipitated with trichloroacetic acid. Controls were included in which water replaced asparagine or extract. The tubes were incubated for 5 hr. in air at c. 18°.

Exp. 2. Veronal extract was prepared from dried cells and dialysed for 2 hr. against running tap water. Conditions and controls as in exp. 1, except incubation was continued for only 60 min.

Exp. 3. Acetone powder (350 mg.) was suspended in 8 ml. of 0.067 M-phosphate (pH 8) and 1 ml. of this suspension was then added to each tube along with 1 ml. of 0.067 M-phosphate (pH 8), 0.5 ml. of 0.1 M-L- β -asparagine and water to 3 ml. The tubes were shaken in air at c. 18°.

Exp. no.	L- β -asparagine disappearing (μ mole)	Ammonia formed (μ mole)	L-aspartic acid formed (μ mole)
1	-33.9	+32.5	Not tested
2	-8.3	+8.1	Not tested
3	-9.3	+8.6	+8.6

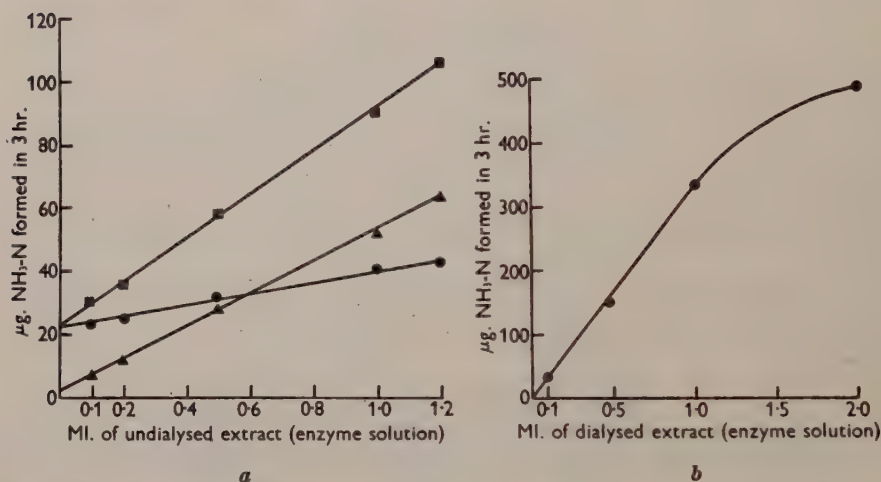
The activity of the asparaginase was extremely variable (cf. Figs. 1a, b). The probable explanation is that the organisms were grown at temperatures ranging from 15 to 30°. The activity was consistently low during the summer and in winter about 5–6 times higher. Both temperature and age of culture have a pronounced effect upon the activity of asparaginase in *Aspergillus niger* (Bach, 1929a; Schmalfuss & Mothes, 1930).

Characteristics of asparaginase in Microsporum gypseum

An asparaginase preparation was obtained from acetone powders of *M. gypseum* by extracting them with 0.1 M-veronal, pH 9. Tenth-molar veronal solution was added to the acetone powder in the proportion of 1 ml./44 mg. powder, the mixture was stirred by hand for 30 min., centrifuged at c. 4000 r.p.m. and used directly or after dialysis for a few hours against running tap water. The protein concentration of such extracts was about 10–12%. Asparaginase could not be extracted with water (Table 5). The requirement of a slightly alkaline reaction for optimal extraction of asparaginase has also been observed with yeast (Grassmann & Mayr, 1933); Geddes & Hunter (1928) found simple

aqueous extracts of yeast were inactive, but they were able to extract the enzyme with 50 % (v/v) glycerol at pH 7 in 3 days.

Figs. 1a and b show graphically the results of two experiments in which the rate of deamidation was proportional to the amount of enzyme present. Diminution of rate shown in curve b (Fig. 1) at the highest concentration of enzyme was probably due to suboptimal concentration of substrate. Fig. 2 shows that initially the reaction rate was constant, but after 3 hr. it decreased. No decisive explanation can be given for this decrease. Attempts to reverse the reaction have so far given equivocal results, so that inactivation of the enzyme seems probable.



Figs. 1a, b. Proportionality between rate of hydrolysis of asparagine and asparaginase concentration as shown by the ammonia formed from asparagine in the presence of varying amounts of a veronal extract of acetone powder.

Fig. 1a. The tubes contained 0.067 M-phosphate buffer (pH 8; 1 ml.), indicated portions of a veronal extract which contained 11.3 mg. protein/ml., 50 μ mole L- β -asparagine in water (0.5 ml.) or water (0.5 ml.) in a final volume of 3 ml. The tubes were incubated for 3 hr. in air at 22°. Extract without asparagine (●—●); extract with asparagine (■—■); difference curve (▲—▲).

Fig. 1b. The tubes contained the indicated portions of veronal extract dialysed 2 hr. against running tap water. Conditions as in Fig. 1a. The values on the curve have been corrected for the ammonia nitrogen formed in the absence of asparagine.

Study of the effect of pH value on the activity of asparaginase in dialysed veronal extracts showed there is a broad optimal pH range between 7.8 and 9 and a maximum close to pH 8.5. Busch (1942) and Geddes & Hunter (1928) found the optimal pH for yeast asparaginase very close to 8. Bach (1929b) found it at 8.6–8.4 for *A. niger*, while Schmalfuss & Mothes (1930) found it at pH 7.7–7.8 for the same organism.

A typical curve relating activity of the enzyme to concentration of L- β -asparagine is given in Fig. 3. The enzyme has a high affinity for its substrate,

half maximum velocity of the enzyme in a veronal extract with 12.8 mg. protein/ml. being at *c.* 0.001 M-asparagine.

As might be expected, since the reaction appears to involve only simple hydrolysis of the amide bond of asparagine, the reaction occurred as well anaerobically as aerobically. For example, using 35 mg. of acetone powder suspended in phosphate buffer pH 8, 38 μ g. of ammonia nitrogen were produced aerobically from asparagine and 44 μ g. anaerobically.

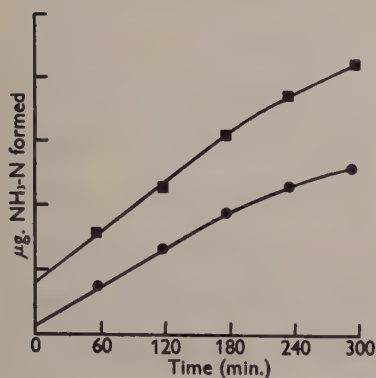


Fig. 2

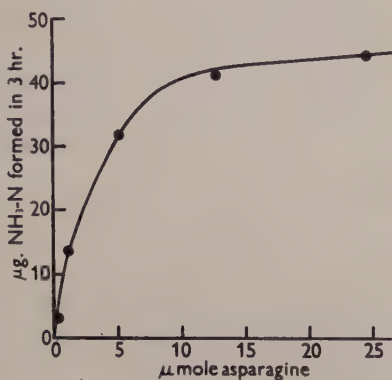


Fig. 3

Fig. 2. Time course of asparaginase action. Conditions as in Table 6, Exp. 3. The tubes were incubated for the indicated periods of time in air at 22°. Corrected (●—●) and not corrected (■—■) for ammonia nitrogen formed in the absence of asparagine.

Fig. 3. Proportionality between the rate of hydrolysis of asparagine by asparaginase in a veronal extract of acetone powder and substrate concentration. The tubes contained 1 ml. of veronal extract (12.8 mg. protein/ml.), 0.02 M-phosphate (pH 8), and indicated portions of L- β -asparagine in a final volume of 3 ml.; the tubes were incubated at 22° for 3 hr.

DISCUSSION

Since the dermatophytes can grow on a mixture of amino-acids or even keratin as sole carbon source (Nickerson, 1947), it seems likely that oxidative deamination of amino-acids is an important step in their utilization. The rate of oxidative deamination observed in acetone powders seems to be too low for the reaction to be essential in the metabolism of the amino-acids. However, as pointed out, the measurement of activity has not been observed under optimal conditions. The relation of asparaginase to the metabolism of *M. gypseum* is similarly difficult to assess. The variability of its activity suggests that its importance to the fungus may change with the age of the culture and the environmental conditions.

The author wishes to express her appreciation to Dr R. Riddell for his encouragement and interest in this investigation.

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(Received 2 September 1952)

Sulphate Reduction in Partially Sterilized Soil Exposed to Air

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SUMMARY: H_2S was evolved from soil treated with CCl_4 when moistened with sucrose and $(NH_4)_2SO_4$ solution and incubated aerobically. H_2S formation took place when the soil moisture was less than field capacity, and over a range of pH values from 5 to 8. The organism responsible was isolated and identified as *Bacillus megaterium*. Several strains of this organism reduced sulphate in well-aerated sterilized soil and liquid media but not in soil or liquid incubated anaerobically.

The action of CCl_4 in fresh soil is to check or destroy certain fungi and bacteria which normally inhibit sulphate reduction by *B. megaterium*. Some of these organisms were isolated and shown to be sensitive to CCl_4 and to inhibit sulphate reduction by *B. megaterium* in sterilized soil. The isolates did not exhibit antibiotic action when grown in certain defined media.

The reduction of sulphate in soil is known to be achieved by *Desulphovibrio* species, and although there have been reports of other micro-organisms able to reduce sulphate (Nadson, 1904; Nastukoff, 1895; Sawjalow, 1913; Shturm, 1948; Tanner, 1918), the widely held view is that only *Desulphovibrio* spp. have been shown with certainty to bring about this reduction (Bunker, 1936; Butlin, Adams & Thomas, 1949; Starkey, 1950; Starkey & Wight, 1945; Young, 1936).

Desulphovibrio spp. are obligate anaerobes and utilize sulphate and other inorganic sulphur compounds as specific hydrogen acceptors during the oxidation of organic energy sources (Baars, 1930). The sulphur compounds are reduced to sulphides. Shturm (1950) claims to have obtained growth of *Desulphovibrio* spp. under aerobic conditions in meat extract broth without added sulphates, but the reduction of sulphate under aerobic conditions has not been reported in the literature as far as is known to the author. The present author observed that soil after treatment with CCl_4 released H_2S from sucrose + $(NH_4)_2SO_4$ solution under what appeared to be well-aerated conditions. When this observation is considered in the light of existing literature the problem arises of how sulphate reduction can occur under such conditions. This paper describes experiments to discover whether anaerobic conditions were developing in the apparently well-aerated soil, the nature of the micro-organisms responsible for the sulphate reduction and the effect of CCl_4 on the process.

METHODS

The soils listed in Table 1 were crushed with pestle and mortar and passed through a nest of sieves of 3, 2, 1 and 0.5 mm. mesh. The particles remaining on the 3 mm. mesh were discarded and the particles on the 2, 1 and 0.5 mm. mesh collected and kept separate. The size of the particles of soil referred to

below are defined by the diameter of the mesh which held them back; thus soil composed of particles which will pass through 3 mm. but not 2 mm. holes is referred to as 2 mm. soil.

Hydrogen sulphide evolution was detected with lead acetate paper. For pH determinations 5 or 10 g. of soil were suspended in 12.5 or 25 ml. distilled water respectively and shaken 20 times every $\frac{1}{4}$ hr. during 2 hr., after which the pH value was obtained by means of a glass electrode. Soil was sterilized by autoclaving 5 g. lots of dry sieved soil in 100 ml. conical flasks for 30 min. at 20 lb./sq.in.

Culture media

Sucrose ($(\text{NH}_4)_2\text{SO}_4$ solution. Sucrose, 5.0 g.; $(\text{NH}_4)_2\text{SO}_4$, 1.25 g.; distilled water, 100 ml.; autoclaved 10 min. at 10 lb./sq.in. This solution was only used for the Rothamsted soil and was usually applied in the proportion of 2 ml./5 g. soil. For other soils the amounts of sucrose and $(\text{NH}_4)_2\text{SO}_4$ in 100 ml. of water depended on the water-holding capacity of the soil.

Sulphate reduction medium (Baars, 1930). K_2HPO_4 , 0.05 g.; sodium lactate (70 % solution), 0.50 g.; NH_4Cl , 0.10 g.; CaSO_4 , 0.10 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.20 g.; $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$, 0.05 g.; tap water, 100 ml.; pH 7.2; autoclaved 15 min. at 15 lb./sq.in.

Czapek agar, modified. NaNO_3 , 0.2 g.; KCl , 0.05 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 g.; KH_2PO_4 , 0.1 g.; distilled water, 100 ml.; sucrose, 3.0 g.; agar, 2.0 g.; pH 4.2; autoclaved 10 min. at 10 lb./sq.in.

Sucrose phosphate peptone agar (SPP). Sucrose, 0.5 g.; K_2HPO_4 , 0.5 g.; Difco peptone, 0.5 g.; distilled water, 100 ml.; agar 2.0 g.; pH 7.5; autoclaved 15 min. at 15 lb./sq.in.

Medium (M) for H_2S evolution by Bacillus megaterium. KH_2PO_4 , 0.5 g.; NaCl , 0.02 g.; CaCl_2 , 0.01 g.; $(\text{NH}_4)_2\text{SO}_4$, 1.25 g.; sucrose, 5.0 g.; distilled water, 100 ml.; Difco yeast extract, 0.3 g.; CaCO_3 , 0.1 g.; pH 6.5; autoclaved 15 min. at 15 lb./sq.in.

Medium for testing antagonisms. Sucrose, 1.0 g.; $(\text{NH}_4)_2\text{SO}_4$, 0.2 g.; Difco yeast extract, 0.05 g.; KH_2PO_4 , 0.05 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g.; distilled water, 100 ml.; agar, 2.0 g.; pH 6.5; autoclaved 15 min. at 10 lb./sq.in.

RESULTS

Conditions under which H_2S evolution occurs in CCl_4 -treated soil

The effect of sucrose and $(\text{NH}_4)_2\text{SO}_4$ concentration on the evolution of H_2S . Ten g. samples of 1 mm. mesh Rothamsted soil were spread evenly over the bottom of 100 ml. conical flasks. Duplicate flasks received the following amounts of sucrose and $(\text{NH}_4)_2\text{SO}_4$ made up in 3.6 ml. distilled water:

$(\text{NH}_4)_2\text{SO}_4$	0.05 g.	0.025 g.	0.005 g.	0.0025 g.
Sucrose	0.2 g.	0.1 g.	0.02 g.	0.01 g.

All the eight flasks then received 1 ml. CCl_4 and strips of lead acetate paper were hung in the flasks to within 1 cm. of the soil surface. After 7 days' incubation at 30° H_2S was detected in all flasks except those containing 0.01 g. sucrose and 0.0025 g. $(\text{NH}_4)_2\text{SO}_4$.

By keeping the sucrose at 0.2 g./flask and adding concentrations of $(\text{NH}_4)_2\text{SO}_4$ decreasing from 0.05 g./10 g. soil to zero, it was found that H_2S was evolved from all cultures except those to which no $(\text{NH}_4)_2\text{SO}_4$ was added. Similarly, by keeping the $(\text{NH}_4)_2\text{SO}_4$ at 0.05 g. and adding sucrose in concentrations ranging from 0.2 g./10 g. soil to zero, H_2S was evolved from all flasks except those containing no added sucrose. When the $(\text{NH}_4)_2\text{SO}_4$ was replaced by NH_4Cl , no H_2S was evolved. This result, together with the observation that no H_2S appeared when $(\text{NH}_4)_2\text{SO}_4$ was omitted, indicated that the sulphur in the H_2S came from the sulphate. An experiment described later supports this view.

Effect of soil type. To observe the effect of soil type on H_2S evolution, soils differing in texture and pH value were treated as follows: duplicate 10 g. samples of 1 mm. soil particles were spread evenly over the bottom of 100 ml. conical flasks and each was given 0.2 g. sucrose and 0.05 g. $(\text{NH}_4)_2\text{SO}_4$ in aqueous solution. The actual volume and hence the strength of the solution added varied with the soil. Each flask was then treated with 1 ml. of CCl_4 . The texture, amount of solution added, the evolution of H_2S and the initial and final pH of the soils after 7 days' incubation at 30° , are given in Table 1. The Old Kennington and Rothamsted soils showed marked evolution of H_2S , Bones Close and Harpenden Common a trace, and Derby and Exton Park soils none.

Table 1. *Changes in pH values and presence of H_2S evolution from various soils moistened with sucrose + ammonium sulphate solution*

				Incubation for 7 days at 30° .		Final pH value		H_2S produced (+) or not (-)	
Soil	Texture	Volume of solution added (ml.)	Initial pH value	Control soil	Soil treated with CCl_4	Control soil	Soil treated with CCl_4		
Old Kennington, Berks.	Sandy clay	2	6.3	5.7	6.4	+	+		
Rothamsted, Herts.	Clay	4	6.5	6.8	6.5	—	+		
Bones Close, Rothamsted, Herts.	Clay	4	7.0	7.0	7.0	—	±		
Harpenden Common, Herts.	Clay	3.5	4.0	3.5	3.4	—	±		
Exton Park, Rutland	Clay	4	7.2	7.1	7.2	—	—		
Derby	Sandy loam	3	4.8	4.7	4.3	—	—		

Effect of pH value. To find the pH range at which H_2S was evolved from Rothamsted soil 1 ml. distilled water and 0.25–2.0 ml. of N-HCl or N-NaOH were added to duplicate 5 g. samples of soil. The soil samples were allowed to dry at 40° for 48 hr. and then treated with sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution and CCl_4 . After 4 days' incubation at 30° the evolution of H_2S was noted and pH determinations made on the soils. Hydrogen sulphide was evolved between pH 5.0 and 7.7 and was greatest between pH 5.5 and 7.0. From the results of this and the previous experiment it was difficult to see why H_2S should not be

produced in Exton Park soil and to a greater degree in Bones Close soil. It was realized that factors affecting H_2S evolution could be different from those affecting sulphide formation, and that the H_2S once produced might form a non-volatile sulphide.

Effect of CaCO_3 . It was noticed that Exton Park and Bones Close soils contained visible amounts of CaCO_3 and that Rothamsted and Old Kennington soils did not. To see what effect this CaCO_3 might have on the evolution of H_2S from Rothamsted soil, increasing quantities of CaCO_3 were added to duplicate 5 g. soil samples; the soils were then treated with sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution and CCl_4 , incubated for 4 days at 30° and then examined for H_2S evolution and their pH values determined. As little as 0.05 g. CaCO_3 /5 g. soil decreased the evolution of H_2S and 0.2 g. prevented it; the pH values of the treated soils were between 7.0 and 7.5.

To see whether sulphide was present in the soils receiving 0.2, 0.3 and 0.5 g. CaCO_3 the suspensions used for pH determinations were acidified with concentrated H_2SO_4 , and in all cases sufficient H_2S was released to darken lead acetate paper. Soils which did not receive sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution did not give a positive test for H_2S on acidification. To see whether H_2S evolution could be obtained from Exton Park soil, 1.5 ml. 2N-HCl was added to 5 g. of the soil to decrease the amount of CaCO_3 , the soil allowed to dry at 40° and then sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution and CCl_4 applied; after 4 days at 30° , H_2S was evolved.

The effect of soil particle size, moisture content and amount of soil. The effect of particle size on H_2S evolution was studied by spreading evenly duplicate 5 g. samples of 2, 1 and 0.5 mm. mesh Rothamsted soil over the bottoms of 100 ml. conical flasks and treating them with sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution and CCl_4 . H_2S was evolved in all flasks after 4 days at 30° .

The effect of moisture content was studied as follows. To duplicate 10 g. samples of air-dried 1 mm. mesh with Rothamsted soil in 100 ml. conical flasks, increasing volumes from 0.5 to 8.0 ml. of sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution were added. This experiment did not test the effect of moisture content alone, as the quantities of sucrose and $(\text{NH}_4)_2\text{SO}_4$ also varied. However, even in 0.5 ml. of the sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution, there was 0.05 g. sucrose and 0.006 g. $(\text{NH}_4)_2\text{SO}_4$ which, from the experiment recorded above, was sufficient to allow H_2S evolution.

The soils which received from 0.5 to 2.0 ml. of solution were shaken to mix the wet and dry soil thoroughly, and then all soils received CCl_4 . H_2S was evolved from all soils except those which received 0.5, 0.75 and 8.0 ml. of solution. The air-dried soil contained 2.5% moisture, and the soil when at field capacity contained 25% moisture. H_2S was evolved from Rothamsted soil which contained as little as 12.5% moisture.

The effect of varying the amount of soil per flask was studied by spreading evenly 1 mm. mesh Rothamsted soil in duplicate amounts of 1–20 g. over the bottoms of 250 ml. conical flasks. The soils were moistened with sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution in the proportion 0.4 ml./g. soil and then treated with 1.0 ml. of CCl_4 . H_2S was detected in all flasks after 4 days at 30° .

Organisms responsible for sulphate reduction

An examination of the microflora in soil after treatment with CCl_4 . It was possible that the CCl_4 may have stimulated the sulphate-reducing bacteria (*Desulphovibrio* spp.). To ascertain whether this were so, soil treated with sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution and CCl_4 , and soil treated with sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution only, were incubated until H_2S was detected in the CCl_4 -treated soil (2 days at 30°), after which time serial dilutions from $1/10$ to $1/10^7$ of both soils were made in sterile water. Baars's sulphate reduction medium in test tubes and as agar plates was inoculated with 1 ml. of each of the dilutions and incubated under hydrogen in anaerobic jars for 10 days at 30° . With either soil treatment H_2S was found in liquid medium only when given $1/10$ dilution of the soil.

To obtain some indication of the qualitative effect of CCl_4 on the microflora the following experiment was conducted. Two 10 g. samples of soil were each moistened with 4 ml. sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution, and one soil sample received in addition 1 ml. CCl_4 . They were incubated at 30° for 48 hr. and H_2S appeared in the CCl_4 -treated soil after this time. To each soil sample 100 ml. sterile-distilled water was added and the samples shaken 100 times, allowed to stand for 10 min. and then shaken another 100 times. Serial dilutions up to $1/10^7$ were made from each suspension. The second shaking after 10 min. was necessary because the soil which did not receive CCl_4 was difficult to wet and remained in clumps bound by fungal growth after the first shaking. One ml. of dilutions of each soil sample were pipetted aseptically into sterile Petri dishes and mixed with melted (45°) sterile Czapek agar or SPP agar. The SPP agar plates were incubated aerobically and anaerobically at 30° and the Czapek plates aerobically at 30° . A general description of the growth developing on these plates is given below. Platings from the CCl_4 -treated soil showed no fungi, few iridescent bacterial colonies and many white opaque colonies on the aerobic plates, and very little growth or gas formation on the anaerobic plates. Platings from the untreated soil showed abundant fungi, iridescent and opaque bacterial colonies on the aerobic plates. Iridescent and opaque colonies also appeared on the anaerobic plates and were accompanied by abundant gas formation.

To determine whether the organisms responsible for the H_2S evolution from the CCl_4 -treated soil were developing on these two media, loopfuls of growth from various aerobic plates were suspended in sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution, and 2 ml. of each of these suspensions were used to inoculate duplicate 5 g. samples of sterile Rothamsted soil. Two soil samples in duplicate were inoculated from each suspension, and one of these soil samples also received 1 ml. CCl_4 . The results after aerobic incubation are given in Table 2.

H_2S was evolved from sterile soil inoculated with organisms from plates which received dilutions of CCl_4 -treated soil, irrespective of whether the inoculated soil received CCl_4 or not. The addition of CCl_4 delayed the appearance of H_2S by 24 hr. Sterile soil inoculated with the organisms from plates which received dilutions of untreated soil only showed strong evolution of H_2S when

the soil received CCl_4 . In the absence of CCl_4 these soils had a mat of fungal mycelia over their surfaces and gave only a slight trace of H_2S .

Thus the organisms responsible for H_2S evolution and the organisms which inhibit H_2S evolution both developed on these media; CCl_4 destroyed inhibitory organisms, which may be fungi or CCl_4 -sensitive bacteria.

Table 2. *Hydrogen sulphide production from sterilized Rothamsted soil moistened with sucrose + ammonium sulphate solution, + or - CCl_4 , and inoculated with organisms from various plates as shown below*

Part 1 Source of inocula for Part 2			Part 2 Sterile Rothamsted soil and nutrients + or - CCl ₄			
Soil and sucrose + (NH ₄) ₂ SO ₄ solution with (+) or without (-) CCl ₄ ; 30° for 48 hr.	Suspension dilution (reciprocal)	Medium plates on 30° aerobic	Time after inoculation with organism from plates of Part 1 aerobic, 30° (hr.)			
			+		-	
			24	48	24	48
H ₂ S production (+ or -)						
+	10 ⁴	Czapek	+	+	-	+
+	10 ⁵	Czapek	+	+	-	+
+	10 ⁵	SPP	+	+	-	+
+	10 ⁶	SPP	+	+	-	+
-	10 ⁴	Czapek	-F	-F	-	±
-	10 ⁵	Czapek	-F	-F	-	±
-	10 ⁶	SPP	-F	-F	-	+
-	10 ⁶	SPP	-	-	-	+

F=fungal growth on soil surface.

Isolation and identification of the H_2S -producing micro-organisms. From previous experiments it was found that the organism which produced H_2S grew aerobically at 30° on SPP agar and survived CCl_4 treatment. Enrichment cultures were obtained in the following manner: 5 g. fresh Rothamsted soil were treated with 1 ml. CCl_4 and moistened with 2 ml. sucrose + (NH_4) $_2\text{SO}_4$ solution. The mixture was incubated at 30° for 3 days, suspended in 20 ml. sterile-distilled water and a loopful of the suspension plated on SPP agar. After 48 hr. at 30° a loopful of growth from the plate was suspended in 2 ml. sucrose + (NH_4) $_2\text{SO}_4$ solution and added to 5 g. sterile Rothamsted soil. The inoculated soil was treated with 1 ml. CCl_4 and incubated until H_2S was evolved. The re-inoculation procedure was repeated three times and from the plating of the suspension from the final enrichment flask, single colonies of different characteristics were picked off and were replated on SPP agar to test purity. Suspensions of these isolates in sucrose + (NH_4) $_2\text{SO}_4$ solution were used to inoculate samples of sterile soil with and without CCl_4 . Of five isolates tested two produced H_2S . In soil cultures of these two isolates the evolution of H_2S appeared 24 hr. later when CCl_4 was added.

Identification of the isolates. The two organisms were similar in biochemical reactions. One was identified as *Bacillus megaterium* (Smith, Gordon & Clark, 1946).

Ten strains of *B. megaterium*, nos. 7851, 2605, 2607, 5635, 5636, 5637, 6007, 6095, 6347, 6005, 6094; two of *B. cereus*, nos. 8035, 7587, and two of *B. mycoides*, nos. 926, 7586, were obtained from the National Collection of Type Cultures. The ability of each of these strains to produce sulphide from $(\text{NH}_4)_2\text{SO}_4$ was tested on sterile soil. The soil was moistened with a suspension of each strain in sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution, incubated at 30° and tested for H_2S evolution. H_2S was evolved from soil inoculated with all strains of *B. megaterium* but not from soil inoculated with the *B. cereus* or *B. mycoides* strains.

Experiments with pure cultures of Bacillus megaterium

Reduction of sulphate. An experiment was made to see whether the sulphur of the H_2S originated from the added sulphate. Radioactive sulphur as $(\text{NH}_4)_2^{35}\text{SO}_4$ was added to the sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution so that 2 ml. of solution contained 5 μc . of ^{35}S . The solution was autoclaved, inoculated with *B. megaterium* and 2 ml. of it added to triplicate 5 g. samples of sterile Rothamsted soil contained in wide-mouthed 100 ml. conical flasks. The lead acetate paper which collected the radioactive H_2S was in the form of a 1.5 cm. diam. disk with a thin strip 6 cm. long projecting from it. The strip of paper was inserted into a piece of glass tubing so that the disk of paper protruded from one end of the tube. The other end of the tube was wrapped centrally inside a cotton-wool plug. The moist lead acetate paper when mounted in this way could be inserted and withdrawn without touching the inside of the flask and thereby avoided possible contamination of the paper with radioactive sulphate. The flasks were incubated for 24 hr. at 30° . The disks of lead acetate paper were completely black after this period.

The disks were removed, dried and then counted; the mean count rates were 993, 960 and 628 counts/min. To obtain some indication of the amount of sulphide trapped by the lead acetate paper, disks of filter-paper were moistened with 0.015, 0.02 and 0.05 ml. of the 5 μc . ^{35}S solution, dried and counted. The mean count rates were 1004, 1367 and 2727 counts/min. respectively. The count rate of the lead sulphide disks approximated to that of 0.015 ml. of original solution which thus represented nearly 0.75 % of the added ^{35}S .

The effect of calcium carbonate, soil pH value and low moisture content on hydrogen sulphide evolution. The experiments on the effect of CaCO_3 , pH value and moisture content on H_2S evolution, from unsterile soil treated with CCl_4 and sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution, were repeated on sterile Rothamsted soil inoculated with a suspension of *B. megaterium* in sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution; 0.05 g. added CaCO_3 /5 g. soil greatly decreased the amount of H_2S evolved, and 0.2 g. CaCO_3 /5 g. soil prevented it. When these cultures were acidified with H_2SO_4 , H_2S was released in sufficient amounts to darken lead acetate paper. The pure culture of *B. megaterium* evolved H_2S from sterile soil at pH values between 5 and 8 and gave the greatest evolution between pH 5.5 and 7.0. Hydrogen sulphide was evolved from 10 g. sterilized soil moistened with as little as 1 ml. sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution and inoculated with *B. megaterium*. These results are in agreement with those obtained earlier with the CCl_4 -treated fresh soil.

Reduction of sulphate in soils of high moisture content. The evolution of H_2S was studied in soils of high moisture content. Duplicate 10 g. samples of sterile Rothamsted soil in 100 ml. conical flasks were moistened with 5 ml. *B. megaterium* suspension in sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution, and also received 1–5 ml. sterile distilled water; another set of 10 g. samples of Rothamsted soil received 10–20 ml. of the *B. megaterium* suspension; a final set received 10 ml. suspension and 10 ml. sterile distilled water.

The soils which received 6–10 ml. of liquid were completely saturated but had soil crumbs projecting above the surface of the solution; those which received 15 and 20 ml. had depths of 2–3 and 4–5 mm., respectively, of liquid above the soil.

H_2S was evolved in sufficient amounts to blacken lead acetate paper completely from all flasks except those in which the soil was completely submerged. In the latter the edges of the lead acetate paper only turned a faint brown. There was no increase in the colour of the lead acetate paper after acidification of the cultures in these flasks.

Reduction of sulphate in liquid media. An attempt was made to obtain H_2S evolution from liquid media. The media were dispensed in duplicate 3 ml. lots in 100 ml. conical flasks, and a strip of lead acetate paper was suspended in each flask. One drop of a suspension of a 48 hr. SPP agar slope culture of *B. megaterium* was added to each flask and the contents incubated aerobically or anaerobically for 4 days at 30° . A number of different media were tested and only medium M was found to give H_2S evolution under aerobic incubation. No H_2S was evolved when Difco yeast extract was omitted or replaced by amino-acids or when $(\text{NH}_4)_2\text{SO}_4$ was replaced by NH_4Cl . Baars's medium was unsuitable for sulphate reduction by this organism.

Aeration experiments. Sterile Rothamsted soil was inoculated with a suspension of *B. megaterium* in sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution and incubated: (a) *anaerobically* for 7 days under hydrogen in a McIntosh & Fildes jar; (b) *aerobically* (1) in a conical flask stoppered with a cotton-wool plug, (2) in a conical flask with a stream of water-washed air blowing on to the surface of the soil, (3) in a glass tube in which a layer of soil 1 cm. thick was supported on glass-wool half-way up the tube and water-washed air was passed through it from below. The water-washing of the air was to remove dust and to minimize evaporation from the soil. The air was delivered from an aquarium aeration pump at a rate of 100 ml./min. The soil which was incubated anaerobically for 7 days did not release H_2S but did so when subsequently incubated aerobically for another 2 days. With the three aeration treatments enough H_2S was released completely to blacken the lead acetate papers.

B. megaterium was plated on SPP agar and incubated aerobically or anaerobically. The anaerobic plates showed no growth whilst the aerobic plates showed abundant growth; the anaerobic plates developed abundant growth after a further 24 hr. aerobic incubation. To test for the presence of *Desulphovibrio* spp. as contaminants in the 'sterilized' soil and *B. megaterium* culture, sterilized soil and fresh soil were moistened with sterile Baars's medium and with Baars's medium inoculated with *B. megaterium*. The soils were incubated

aerobically and anaerobically for 10 days at 30°. H_2S was evolved only from the anaerobic fresh soil, which indicated that both the sterilized soil and the culture were free from *Desulphovibrio* spp.

The effect of carbon tetrachloride on the evolution of hydrogen sulphide from soils

Comparison with other volatile substances. Duplicate 5 g. samples of Rothamsted soil were treated with 2 ml. of one of the following substances: diethyl-ether, CCl_4 , chloroform, toluene, xylene, 95 % (v/v) ethanol, benzene; or with 0.8 ml. of 2 % (v/v) phenol or 40 % formalin. H_2S was evolved from those soils treated with ether, CCl_4 , chloroform or benzene. Similar experiments were repeated, but instead of using equal volumes of the volatile compounds the volumes used were the same as those lost by evaporation after 24 hr. These volumes were estimated by measuring changes in volume due to evaporation from a free surface 1 in. in diameter at hourly intervals. In this case the soils treated with ether, CCl_4 , chloroform, toluene, xylene or benzene all produced an increase in H_2S evolution. A further attempt was made to get a similar effect with formalin, ethanol and phenol. Duplicate 5 g. samples of air-dried Rothamsted soil in Petri dishes were treated with 1 ml. of 1 % formalin, 2 % (v/v) phenol or 95 % ethanol, or subjected to the vapours from filter-paper moistened with 0.1 ml. 40 % formalin. The soils remained open to the air until the ethanol and formalin could not be detected by smell and until the phenol-treated soil was air dry. The soils were then moistened with sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution and tested for H_2S . Only the ethanol-treated soils evolved H_2S on incubation.

To determine whether H_2S was evolved while CCl_4 was present or only after it had evaporated, three sets of flasks in duplicate containing 5 g. Rothamsted soil were treated with sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution and 2 ml. CCl_4 . The first set received no further treatment; the second had fresh lead acetate paper added every 24 hr.; the third had an additional 3 ml. CCl_4 added daily. H_2S was evolved in the first and second sets after 48 hr. incubation and continued to be evolved for another 48 hr. No H_2S was evolved from the third set during 7 days; after this time the daily additions of CCl_4 were stopped and 2 days later H_2S was evolved.

In another experiment, as the amount of CCl_4 was increased from 0.01 to 5 ml./5 g. soil the time for H_2S to be evolved increased from 1 to 8 days and the amount of H_2S increased too, as judged by the degree of darkening of the lead acetate paper. Thus it appeared that the H_2S was released after the CCl_4 had evaporated. H_2S was evolved from heated soil, heated at 70° for 1 hr. whether or not CCl_4 was added, but not from autoclaved soil. Thus it appeared that the effect of CCl_4 on H_2S formation was similar to a partial sterilizing effect.

Inhibition of the H_2S -producing Bacillus by other micro-organisms

Earlier experiments showed that H_2S evolution in soil could be inhibited by CCl_4 -sensitive micro-organisms (see Table 2).

Isolation of inhibitory micro-organisms. In a preliminary experiment it was found that *B. megaterium* would not grow on SPP agar containing 1 part in 750,000 of crystal violet. The following experiment was planned to find whether the organisms which developed on crystal violet SPP agar were CCl_4 -sensitive. Two Rothamsted soil samples received sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution and were incubated for 3 days; one sample also received CCl_4 . Crystal violet SPP and SPP agar plates were inoculated with 1 ml. of $1/10^4$ to $1/10^8$ dilutions of these soil samples. One lot each of crystal violet SPP and of SPP agar plates were flooded with 1 ml. CCl_4 immediately after inoculation and another lot was left untreated. The duplicate plates were incubated at 30° for 96 hr. The growths which resulted from the $1/10^8$ soil dilution is given in Table 3

Table 3. *Growth of SPP agar and crystal violet SPP agar inoculated with $1/10^8$ dilutions of soils after various treatments*

Initial treatment of soil in addition to sucrose + $(\text{NH}_4)_2\text{SO}_4$ solution	Medium inoculated	Treatment given to plate	Type of growth
CCl_4 , 1 ml.	SPP	1 ml. CCl_4	Large white to buff, smooth and rough, shiny and dull, opaque bacterial colonies. No iridescent colonies.
CCl_4 , 1 ml.	Crystal violet SPP	1 ml. CCl_4	No growth
CCl_4 , 1 ml.	SPP	None	Large white buff, smooth, rough, shiny, dull, opaque bacterial colonies. Granular iridescent colonies
CCl_4 , 1 ml.	Crystal violet SPP	None	No growth
None	SPP	1 ml. CCl_4	White, buff, smooth, shiny, dull, rough, opaque colonies and granular iridescent colonies
None	Crystal violet SPP	1 ml. CCl_4	No growth
None	SPP	None	Fungi, white, buff, opaque colonies, large mucoid colonies and non-granular, bluish, iridescent colonies
None	Crystal violet SPP	None	Fungi, large mucoid, some opaque bacterial colonies, also bluish, iridescent colonies

The results for a given treatment were similar at all dilutions. The micro-organisms which developed on crystal violet + SPP agar were CCl_4 -sensitive and the micro-organisms which were CCl_4 -resistant were crystal violet-sensitive. At the above dilutions there were no crystal violet-tolerant micro-organisms in soil treated with CCl_4 , whereas in the untreated soil there were some micro-organisms which were sensitive and some which were tolerant to either crystal violet or CCl_4 . CCl_4 -sensitive bacteria were isolated from the crystal violet + SPP agar plate which received the $1/10^8$ dilution of untreated soil. The isolates

were replated to test their purity and checked for CCl_4 sensitivity in the following manner; SPP agar was plated with the isolate, flooded immediately with 1 ml. CCl_4 and incubated, after the CCl_4 had evaporated, for 72 hr. at 30° and then examined for growth. Fungi were isolated from the $1/10^4$ dilution of the same soil on Czapek agar and tested for CCl_4 sensitivity in the same way. In the above manner six cultures of CCl_4 -sensitive bacteria and four cultures of CCl_4 -sensitive fungi were obtained.

*The effect of CCl_4 -sensitive micro-organisms on H_2S evolution by *B. megaterium*.* To determine whether these micro-organisms inhibited the evolution of H_2S , 5 g. samples of sterile Rothamsted soil were inoculated with a mixed suspension of *B. megaterium* and the micro-organisms to be tested; some of the soil samples also received CCl_4 . A micro-organism was considered to be inhibitory when H_2S was evolved from the CCl_4 -treated soil but not from the untreated soil to which it had been added. The four fungi and six CCl_4 -sensitive bacteria were tested in this way against *B. megaterium*; one fungus and three bacteria inhibited the evolution of H_2S . The fungus was identified as a member of the genus *Mucor*, two of the inhibiting bacteria as species of *Pseudomonas*, and the other a species of *Bacterium*. One of the CCl_4 -sensitive bacteria which did not inhibit H_2S formation by *B. megaterium* belonged to the genus *Achromobacter*, and the other two to the genus *Aerobacter*. Thus the inhibitory bacteria did not belong to the same species or even the same genus and not all the CCl_4 -sensitive bacteria inhibited the formation of H_2S by *B. megaterium*.

An attempt was made to see whether the inhibition of H_2S evolution was due to an antibiotic effect of the fungus and one of the bacteria on the *B. megaterium*. Plates of sucrose + $(\text{NH}_4)_2\text{SO}_4$ medium were inoculated, either over the whole surface with a mixture of the test organism and *B. megaterium* or inoculated in straight lines with the inhibitory organism at right angles to the *B. megaterium*, and incubated for 5 days at 30° . No antagonism was observed on the plates inoculated by streaking. On the mixed inoculum plates the *B. megaterium* formed slightly smaller colonies when grown in the presence of the bacterium as compared with the colonies of the pure culture of *B. megaterium*; the fungus showed no inhibition on the mixed plates.

*The effect of phenol, formalin and ethanol on *B. megaterium* and on the inhibitory micro-organisms.* Phenol, formalin and ethanol are known to have a partial sterilizing effect when applied to soil, but in the experiment described earlier phenol, formalin or ethanol in the presence of water did not exhibit the same partial sterilizing effect as CCl_4 . To see what effect these substances had on *B. megaterium* and the inhibitory organisms, SPP agar plates were inoculated with these micro-organisms and flooded immediately after with 1 ml. of 2% phenol, 1% formalin or 95% alcohol, and examined for growth after 7 days' incubation at 30° .

The inhibitory bacteria and the fungi were killed by all three solutions, *B. megaterium* only by formalin. *B. megaterium*, however, failed to grow on SPP agar which contained 0.7% phenol or 30% (v/v) ethanol in water. These results explained why phenol, formalin and ethanol solutions did not behave in the same way as CCl_4 . Formalin kills *B. megaterium* as well as the inhibitory

micro-organisms; phenol and ethanol, however, because of their miscibility with water, remain in the soil, and although they do not kill the spores of *B. megaterium* they prevent their germination.

DISCUSSION

Previously the biological reduction of sulphate to H_2S has been associated with anaerobic conditions and the activities of anaerobic sulphate-reducing *Desulphovibrio* spp. The results of the present work show that sulphate can be reduced under well-aerated conditions by the aerobic *Bacillus megaterium*. From the fact that sulphide was produced in only one of a number of liquid media it would appear that to get a complete understanding of the activities of soil micro-organisms it is necessary to study their behaviour in soil as well as in laboratory media. The high sugar and $(NH_4)_2SO_4$ concentrations used in the experiments makes it unlikely that this type of reduction occurs to any marked extent in normal soils, but a study of reduction in the presence of other carbon and nitrogen sources might help in assessing its importance in nature.

The part played by CCl_4 is one of partial sterilization. As a result of this, a number of different micro-organisms are killed, and amongst these are some which inhibit the evolution of H_2S by *B. megaterium*. These results indicate that in soil the type of reaction carried out by a particular micro-organism may depend, in part, on the types of accompanying micro-organisms; and that a number of processes normally not active in soil may become intensified as a result of the simplification of the soil microflora by partial sterilization. The way in which some of the CCl_4 -sensitive micro-organisms inhibit sulphate reduction by *B. megaterium* is not known; no antibiotic effect could be demonstrated.

I wish to express my thanks to C.S.I.R.O., Australia, who made this research possible, to Dr H. G. Thornton, F.R.S., for criticism and advice, to Dr Mattingley for his help with radioactive sulphur and to Miss Mabel Dunkley for preparing the typescript.

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(Received 18 September 1952)

COWAN, S. T. (1953). *J. gen. Microbiol.* 8, 391-396.

Fermentations: Biochemical Micromethods for Bacteriology

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SUMMARY: Discrepancies found in the use of three previously published micro-methods for fermentations were traced to at least three possible sources of error: contamination of the water used for making suspensions; contamination of capillary tests from non-sterilizable Perspex plates; chemical breakdown or contamination of certain sugar solutions. To overcome these discrepancies one of the methods was modified; the other two were abandoned.

Suspension concentration is important and, measured as opacity, should not be less than the equivalent of 10^9 *Bacterium coli* cells/ml. Sugar concentration is much less critical, and 0.1 M concentrations are recommended. Dilution of the test mixture with 0.5 ml. sterile water speeds up fermentations; the addition of formalin or thiomersalate inhibits certain enzyme systems but does not hinder glucose fermentation.

Micromethods to test the biochemical activity of bacteria in non-multiplying suspensions were described by Clarke & Cowan (1952); extended trial has shown their usefulness and also some of their limitations. As originally described, the tests used techniques common in biochemical laboratories for experiments of short duration, but their application to bacterial fermentations often demanded prolonged incubation before a final reading was taken. Attention was at first concentrated on single tests on a wide variety of strains, and it was not then obvious that erroneous results were sometimes being obtained; fermentation in a microtest which was not paralleled by fermentation of the same sugar in peptone water was thought to be due to a masking effect of alkaline products from peptone breakdown by the growing culture. Several months later many strains were re-examined, and when, as sometimes happened, different results were obtained in the microtest, our explanation of discrepancies seemed to be false, or, at the best, only a half-truth. This paper records examples of misleading results, explanations of their probable causes, and modifications of technique necessary to avoid their repetition.

EXPERIMENTAL

Example of erroneous result

In the original method (Clarke & Cowan, 1952) growths were washed off agar slope cultures and were resuspended in unsterilized tap water. Usually these suspensions were satisfactory, but at times we observed examples of unexpected acid production in sugar solutions that were chemically satisfactory. In November and December 1951 several strains of *Bacterium alcaligenes*, *Brucella* spp., and *Haemophilus bronchisepticus* were tested and acid was produced, usually after overnight incubation, from many of the

sugar solutions. When retested in January 1952, entirely different results were obtained, and few of the strains showed any fermentative ability. On 5 February 1952, NCTC 655 (*Bacterium alcaligenes*) was examined by the routine microtests; acid was produced from several sugars in the capillary fermentations (method 2 of Clarke & Cowan), but not in open columns (method 3); in other biochemical tests, mostly read within a few hours, the

Table 1. *Routine microtests on NCTC 655 and two contaminating strains, 655/A and 655/B*

	655		655/A		655/B	
	Capillaries	Columns	Capillaries	Columns	Capillaries	Columns
Glucose	A ^{24*}	—	A ⁴	A ⁴	A ⁴	A ⁴
Arabinose	A ²⁴	—	A ⁴	A ⁴	A ⁴	A ⁴
Xylose	A ²⁴	—	A ²⁴	A ²⁴	A ²⁴	A ²⁴
Rhamnose	A ²⁴	—	A ²⁴	A ⁴	A ⁴	A ⁴
Lactose	A ⁴⁸	—	A ⁴⁸	—	A ⁴⁸	—
Sucrose	—	—	A ⁴⁸	—	A ⁴⁸	—
Maltose	A ²⁴	—	A ²⁴	A ²⁴	A ²⁴	—
Trehalose	A ²⁴	—	A ⁴	A ⁴	A ⁴	A ⁴
Glycerol	—	—	A ⁴⁸	A ²⁴	A ⁴⁸	A ²⁴
Mannitol	A ²⁴	—	A ⁴	A ⁴	A ⁴	A ⁴
Dulcitol	A ⁴⁸	—	A ⁴⁸	A ²⁴	A ⁴⁸	A ²⁴
Sorbitol	A ²⁴	—	A ⁴	A ⁴	A ⁴	A ⁴
Inositol	A ²⁴	—	A ⁴	A ²⁴	A ⁴	A ²⁴
Adonitol	—	—	—	—	—	—
Salicin	—	—	A ²⁴	—	—	—
Indole formation	nt	—	nt	—	nt	—
H ₂ S production	nt	—	nt	+	nt	+
Nitrate reduction	nt	—	nt	+	nt	+
M.B. reduction	+	nt	+	nt	+	nt
Catalase	+	nt	+	nt	+	nt
Gelatin hydrolysis	nt	—	nt	—	nt	—
Urease	—	nt	nt	—	nt	—
Starch hydrolysis	nt	—	nt	—	nt	—
Acetoin formation	nt	—	nt	—	nt	—

* Strains 655/A and 655/B were isolated from this tube.

A = acid. Superior figures indicate time (hr.) of reading.

— = Acid not produced, or negative result in other test; + = positive result; nt = no test.

suspension seemed almost inactive. After 24 hr. incubation the capillary tube containing glucose and suspension was opened, the contents were diluted in broth and plated on MacConkey agar and on Lemco agar. Two types of colony (655/A and 655/B) grew on these plates and subcultures were put through the routine microtests. Both cultures were Gram-negative rods, and it was expected that one would be the original strain 655, and the other a contaminant. The results (Table 1) showed that neither colony type was descended from the original culture; that both were active fermenters of sugars in capillaries, open columns and in ordinary peptone-water cultures (not shown in the Table); that both produced H₂S and reduced nitrates to nitrites, characters not shown by NCTC 655. When search was made, similar organisms were found occasionally in our tap water.

Bigger (1937) found that certain coliform organisms could multiply in distilled water, while Allen, Pasley & Pierce (1952) showed that *Bact. coli* grew when the medium was diluted to 10 parts/million parts water. It seemed probable that such an organism when contaminating our suspending fluid could multiply in the residual nutrient materials in our suspensions and ferment various sugar solutions. However, contaminating organisms from water were not the sole, or even the main, source of false fermentations. Table 1 shows that the suspension of 655 used on 5 February did not ferment sugars in the open columns (method 3). In comparative trials greater fermentative ability in capillary tubes was a constant finding; it was attributed, probably wrongly, to an anaerobic type of fermentation. By substituting sterile distilled water for the unsterilized tap water as suspending fluid the results with open columns (method 3) and tubes (method 1) became more consistent, but tests in capillaries continued on occasion to show irregular and unexpected fermentation.

Suspension + sugar mixtures for capillary tests presented a practical problem, namely, cross-contamination of sugar solutions and suspension. To avoid contamination of sugar solutions the capillaries were one-third filled with solution before being dipped into suspension; to avoid carry-over of sugar solution to the suspension, drops of suspension were pipetted into hollows in Perspex plates, one drop for each sugar, and from these drops the filling of the capillaries was completed; they were then two-thirds full and were sealed.

After use the Perspex plates were immersed in dilute HCl (5 vol. conc. HCl + 95 vol. H₂O) for 24 hr., washed in running tap water, drained, and the 80 hollows dried with a glass-cloth, clean on Mondays but soiled by Saturdays. These plates could not be heat sterilized before use, and they appeared to be the source of contaminating organisms responsible for false reactions in capillaries only. As a sterilizable substitute was not readily available, the obvious way to avoid carry-over seemed to be to use small test tubes; this would make a large addition to the washing up, or an extra manipulation to the test in small tubes (method 1). Consequently the capillary method was abandoned and attention directed to speeding up the method which used small tubes.

Other sources of error. Sugar solutions were sterilized either by momentary autoclaving (Davis & Rogers, 1939) or by filtration, and were pipetted aseptically to sterile screw-capped bottles. When tested with Benedict's reagent, autoclaved sucrose solutions contained reducing substances which were absent from the original powdered sugar and from its solution. Sucrose would not tolerate even momentary autoclaving, and solutions were sterilized by Seitz filtration. However, occasional bottles of filtered sucrose showed reducing substances, and it is probable that traces of sugar may remain between the washer and the screw-cap, an experience similar to that of Allison & Smyth (1945) when they used the same type of bottle as a sputum container. To overcome this difficulty new bottles and caps were clearly marked and used only for one sugar solution; metal caps and washers were separated and cleaned individually.

Factors affecting fermentation

Before describing the modification finally developed it will be an advantage to outline the original method 1, and to show by experiment the effect of making changes, small in themselves, on the results. As originally described, method 1 (Clarke & Cowan, 1952) was carried out in 65×10 mm. tubes which contained suspension, 0.2 ml.; sugar solution, 0.1 ml.; buffer + indicator solution (0.025 M-phosphate buffer, pH 6.8, 20 ml.; 1% ethanolic bromocresol purple, 1.0 ml.), 0.2 ml.; and the results were read after incubation in a water-bath at 37° .

Effect of suspension concentration. A suspension of a salmonella species was diluted tenfold, and 0.2 ml. volumes mixed with 0.1 ml. 25% glucose solution or 10% mannitol. The results are shown in Table 2. All experiments in which the effect of suspension concentration was tested made it clear that a suspension was useless unless its opacity was at least as great as that of 10^9 *Bact. coli*/ml.

Table 2. *Effect of suspension concentration and dilution of the test mixture on the speed of acid production from glucose and mannitol by suspensions of cells of a salmonella species*

Suspension concentration (cells/ml.)	Microtest method 1		Method 1 + 0.5 ml. water	
	Glucose	Mannitol	Glucose	Mannitol
	Time to first positive reading (hr.)			
10^{10}	1	3	1	1
10^9	3	21	2	5
10^8	48	—	48	—

— = Indicator not changed.

Effect of dilution of test. Although suspension concentration is most important in these microtests, dilution of the whole test speeds up fermentation. Table 2 shows that the addition of 0.5 ml. sterile distilled water to each tube increased the speed of indicator change for all mixtures. This dilution effect appeared to be the result of weakening the phosphate buffer (Table 3). When the test is diluted with water the effect of the phosphate buffer is negligible, and it was omitted in the other experiments to be described. One other change was made; bromthymol blue was substituted for bromocresol purple so as to detect smaller changes in pH value.

Effect of bacteriostatics. Advantage was taken of the addition of diluent to test the effect of bacteriostatics. Glucose fermentation was almost inhibited by chloroform and toluene but not by suitable concentrations of Nipagin M (methyl-*p*-hydroxybenzoate), thiomersalate or formalin. Thiomersalate and formalin inhibited the fermentation of sucrose and lactose by many coliform organisms; only the fermentation of mannitol and sorbitol were unaffected by formalin.

Effect of sugar concentration. At first high concentrations of sugar were chosen for the microtests, and many solutions were near the limits of solu-

bility of their sugars. Table 4 shows a comparison of results obtained with 52 strains, representative of the different genera in the family Bacteriaceae, in peptone-water cultures and when tested by a micromethod using strong

Table 3. *Effect of dilution of test mixture when sugar concentration is varied*

Addition	Glucose		Maltose		Mannitol	
	25 %	2.5 %	25 %	2.5 %	10 %	1 %
	Time (hr.) to change in colour of indicator					
None	1/4	1/4	3	5	1/2	1/2
Water	1/4	1/4	3	3	1/4	1/4
0.01 M buffer	1/2	1/2	24	24	24	3
0.125 M buffer	3	3	24	24	24	—

— = No change.

Table 4. *Comparison of fermentation reactions of 52 strains of Bacteriaceae in peptone water cultures and in microtests using different concentrations of sugars*

		Micromethods						
		1c			1d			
		Peptone water	Sugar concentration (%)	No. of strains reacting		Sugar concentration (%)	No. of strains reacting	
	Result			+	—		+	—
Glucose	+	52	25	52	0	1.80	52	0
Arabinose	+	48	10	48	5	1.50	39	9
	—	4		1	3		0	4
Xylose	+	39	25	38	1	1.50	36	3
	—	13		5	8		4	9
Rhamnose	+	36	10	28	8	1.82	26	10
	—	16		3	13		2	14
Lactose	+	19	25	18	1	3.60	15	4
	—	33		4	29		3	30
Sucrose	+	14	25	11	3	3.42	11	3
	—	38		2	36		3	35
Maltose	+	43	25	41	2	3.60	40	3
	—	9		6	3		2	7
Trehalose	+	44	10	41	3	3.42	42	2
	—	8		3	5		5	3
Glycerol	+	46	25	46	0	0.92	46	0
	—	6		6	0		6	0
Mannitol	+	43	10	43	0	1.82	43	0
	—	9		3	6		2	7
Dulcitol	+	19	5	14	5	1.82	14	5
	—	33		2	31		2	31
Sorbitol	+	35	10	35	0	1.91	35	0
	—	17		10	7		6	11
Inositol	+	16	5	15	1	1.80	15	1
	—	36		2	34		2	34
Adonitol	+	10	5	8	2	1.52	8	2
	—	42		0	42		0	42
Salicin	+	19	5	13	6	2.86	12	7
	—	33		4	29		3	30

+ = Fermented: — = not fermented.

and weak solutions of sugar. A distinction was not made in the degree of acidity or speed of its production in the peptone-water cultures, which were read after 14 days incubation; microtests were read after 24 hr. From Table 4 the extent of the differences between the growing cultures and micromethods can be seen; it is also evident that the variation in sugar concentration does not have a great effect. The absence of negative tests with glycerol can probably be explained by impurities in the reagent.

Modification of method

During the course of experiments method 1 was modified several times. The final form (method 1d), which seems to give results most in agreement with the usual tests in growing cultures, is as follows:

Suspensions are made in sterile distilled water, pH 7.6–7.8, from growth on a medium not containing a fermentable sugar. The diluting fluid used for the test is sterile distilled water, pH 7.8–8.0, to which is added 5% (v/v) bromthymol blue solution (British Drug Houses Ltd.). Mixtures are made in chemically clean 65 × 10 mm. tubes, and each tube contains, in order of addition: diluting fluid + indicator, 0.5 ml.; 0.1 M sugar solution, 0.05 ml.; suspension, 0.05 ml. Tests are incubated in a water-bath at 37° and readings made after 6 and 24 hr.

DISCUSSION

The evidence presented seems to justify the conclusion that fermentation tests cannot be looked upon simply as a biochemical problem; if pitfalls are to be avoided the methods must use bacteriological precautions to avoid contamination. In principle there is nothing wrong with the capillary method which uses little material and shows results quickly. In practice it is admirable for small-scale work, but for routine use it fails because of the technical difficulty of maintaining a reasonable degree of asepsis when making so many different mixtures. The mixtures can best be made in small test tubes, and to transfer the contents to capillary tubes is an unnecessary manipulation. Dilution of mixtures in the small tubes had the unexpected effect of speeding up the fermentations, and when advantage of this was taken the method was almost as rapid as the capillary test.

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(Received 28 October 1952)

CLARKE, P. H. (1953). *J. gen. Microbiol.* 8, 397-407.

Hydrogen Sulphide Production by Bacteria

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SUMMARY: Hydrogen sulphide production by growing cultures and non-multiplying suspensions was compared and the factors influencing the sensitivity of the tests were investigated. Cysteine hydrochloride (0.01 %) was added to Lemco broth to provide a medium with a standard source of sulphur. H_2S was detected with lead acetate papers more readily than by lead acetate agar.

Suspensions were tested with cysteine, sodium thiosulphate and sodium sulphite; the organisms investigated were mainly Bacteriaceae and seldom failed to produce H_2S from cysteine; sodium thiosulphate was less readily attacked; sodium sulphite was unsuitable for this technique. Media commonly used for testing the capacity of bacteria to produce H_2S are reviewed and the value of this test in bacterial classification discussed.

Hydrogen sulphide was one of the earliest products of bacterial decomposition to be recognized. Orłowski (1895) described H_2S production by the typhoid bacillus, and Burnet & Weissenbach (1915) and Jordan & Victorson (1917) in comparative studies used H_2S production to distinguish between the paratyphoid and enteritidis groups. Sasaki & Otsuka (1912) and Bürger (1914) showed that many different species of bacteria produced H_2S from cystine but not from taurine. Tanner (1917) found that a large number of bacteria produced H_2S from peptone or cystine, and some from thiourea and thiosulphate, though none examined produced it from sulphate or sulphite. Myers (1920) and Tilley (1923*a, b*) found variations in H_2S production from different peptones; Tilley concluded that this was not directly related to the cystine content of the peptone. He tested media reinforced with cystine, thiosulphate, sulphite and sulphate, and concluded that a thiosulphate medium was most useful for diagnostic use. Wilson (1922-3) showed that the typhoid and paratyphoid bacteria could produce H_2S from sulphite in the presence of a fermentable carbohydrate and recommended an iron + sulphite + glucose organic medium. Tarr (1933*a, b*) showed that the production of H_2S from cystine and cysteine by washed suspensions of *Proteus vulgaris* and *Chromobacterium prodigiosum* was enzymic; he obtained a cell-free preparation from *Proteus* (Tarr, 1934). He also found that the production of H_2S by *Proteus* from cysteine and thiosulphate was additive and independent and therefore due to two distinct enzyme systems. The cysteine enzyme was shown to be highly specific and only attacked compounds containing the α -amino- β -thiolcarboxylic acid structure. Desnuelle & Fromageot (1939) obtained a cell-free enzyme preparation from *Bacterium coli* to which they gave the name cysteinase. This enzyme was investigated in a number of species by Desnuelle & Fromageot (1939) and by Desnuelle, Wookey & Fromageot (1940). Fromageot (1951) concluded that cysteinase occurs in all

bacteria which produce H_2S from organic media, and that cystine is first reduced to cysteine.

The numerous methods used to detect H_2S production by micro-organisms vary with the source of sulphur and the metal salts used to indicate H_2S formation. Lead salts were added as indicators in the media of Burnet & Weissenbach (1915), Jordan & Victorson (1917), Kligler (1917), Thompson (1920-1), Morrison & Tanner (1922), Tilley (1923*a*), Bailey & Lacey (1927), Spray (1936) and Friewer & Shaughnessy (1944). Iron was added by Wilson (1922-3), Beckwith & Moser (1932), Levine, Vaughn, Epstein & Anderson (1931-2), ZoBell & Feltham (1934), Tittsler & Sandholzer (1937), Sulkin & Willett (1939-40) and Hajna (1945). Bismuth was used in the selective bismuth sulphite medium for the typhoid-paratyphoid group (Wilson & Blair, 1927; Wilson, 1938) and also by Pacheco & Mello (1932), Hunter, Feldman & Crecelius (1937) and Hunter & Crecelius (1938). Nickel and cobalt salts were introduced by Utermohlen & Georgi (1940), and Beckwith & Moser (1932) used a manganous salt medium on which H_2S producers gave pink colonies. All these metallic ions are toxic to some extent, but metal salts can also be used on strips of paper above the growing culture and lead acetate is the usual choice. Wilmet (1927) showed that his lead acetate papers could detect 1 part H_2S in 7 million of air. Sasaki & Otsuka (1912), Tanner (1917), Myers (1920), Morrison & Tanner (1922) and Huddleson (1929) used lead acetate paper strips, and ZoBell & Feltham (1934), in a study of the sensitivity of methods for H_2S detection by various metal salts in relation to toxicity, concluded that lead acetate papers should be used for testing unknown cultures.

The basal medium used is usually complex, containing meat extract or peptone, and is often reinforced with an additional source of sulphur, either thiosulphate (Tilley, 1923*b*; Tittsler & Sandholzer, 1937; Sulkin & Willett, 1939-40; Hajna, 1945), cysteine (Patrick & Werkman, 1933; Utermohlen & Georgi, 1940), or cystine (Seeliger, 1950). Sulphite is added in the bismuth glucose sulphite agar used for the isolation of *Salmonella typhi* and *Sal. paratyphi* B (Wilson & Blair, 1927; Wilson, 1938; Tabet, 1938; Cook, 1952), and in the bismuth medium of Hunter & Crecelius (1938). Some media contain sugars so that fermentation tests can be carried out simultaneously (Kligler, 1918; Bailey & Lacey, 1927; Sulkin & Willett, 1939-40; Hajna, 1945); some are semi-solid and used for simultaneous determination of motility. Knox (1949) developed a double medium plate which could be used for testing several key biochemical reactions very rapidly. Kauffmann (1951) used a peptone meat-extract medium containing ferric chloride and gelatin for determining H_2S production and gelatin liquefaction. All these media have their uses under the conditions for which they were devised, but, as many authors have shown, the results are not strictly comparable. Discrepancies may be due to the varying sensitivities of the indicators, the ability of the test organism to attack the sulphur source, the amount of growth on a given medium, or the presence or absence of fermentable carbohydrates (Wilson, 1922-3; Heap & Cadness, 1924-5), or other reducing substances.

ZoBell & Meyer (1932) found that with the *Brucella* group the amount of H_2S produced varied with the oxidation-reduction potential. It is probably the slow oxidation of various compounds by the air which causes some H_2S media, particularly the bismuth sulphite plate media, to deteriorate on storage. Vaughn & Levine (1936), using a ferric citrate agar, showed that the number of H_2S -positive coli-aerogenes strains was increased if the agar was decreased in concentration or omitted.

The Society of American Bacteriologists (1947) recommend for use with pure cultures test strips of lead acetate paper over a suitable broth or peptone medium containing an adequate sulphur source. The advantages of this technique are the increased sensitivity and reproducibility of results and the avoidance of any toxic effects of metallic salts in the medium. It was found in this laboratory that variable results were obtained with different batches of Lemco and peptone. The addition of 0.01 % cysteine hydrochloride gave a uniform source of sulphur and clear-cut results.

Clarke & Cowan (1952) devised a test for H_2S production from cystine as part of a scheme for testing the biochemical reactions of non-multiplying suspensions of bacteria with pure substrates. We have now modified the method and use cysteine hydrochloride instead of cystine and detect H_2S with a strip of lead acetate paper. This modified method is less sensitive to variations in cell suspension density. Many strains have been investigated for their ability to produce H_2S from cysteine without the complicating effects of medium variation and compared with the results obtained with the routine medium. A smaller number of strains has been tested with sodium thio-sulphate and sodium sulphite.

EXPERIMENTAL

Organisms. Strains used in this investigation included members of the following genera: *Chromobacterium*, *Proteus*, *Salmonella*, *Shigella* and the coli-aerogenes group of *Bacterium*.

Media. The media used for all the tests on growing cultures were: Lab Lemco (1 %), Evans peptone (1 %), sodium chloride (0.5 %) in water and adjusted to pH 7.6, or this mixture with the addition of 0.01 % cysteine hydrochloride.

For the preparation of suspensions the cultures were grown on Lemco agar slopes unless otherwise described in the text. In this investigation, three to four agar slopes were used for carrying out the complete series of microtests (Clarke & Cowan, 1952). The minimum density of suspensions should be equivalent to 10^8 *Bact. coli* cells/ml.

Microtest. Earlier tests were carried out by the method described by Clarke & Cowan (1952). The later tests and all the tests with thiosulphate and sulphite were carried out as follows: Suspension 0.04 ml., was mixed in 65×10 mm. tubes with 0.1 % cysteine hydrochloride (reaction adjusted to pH 7.4) 0.06 ml., phosphate buffer (0.025 M, pH 6.8) 0.04 ml. A strip of lead acetate paper was held in the mouth of the tube by a cotton-wool plug. The tubes were incubated at 37° either in a water-bath or an air incubator and read at intervals up to

24 hr. Results could usually be read after 15–30 min., but for routine tests 2 and 24 hr. readings were taken. A modification of Almy's method (Almy, 1925) was tried but proved less sensitive than the lead acetate paper method.

Buffers. For the investigation of pH effect on enzyme activity, Walpole's acetate buffers, Sørensen's phosphate buffers, and Clarke & Lubs borate buffers were used.

RESULTS

Microtest with cystine and cysteine. Cystine and cysteine were compared as substrates for the microtest. *Pseudomonas aeruginosa* was slightly more active on cysteine, but *Bact. aerogenes*, *Bact. dispar*, *Sal. enteritidis* and *Sal. typhimurium* gave identical results after 2 hr. on both substrates. Boiled suspensions were completely negative after 24 hr.

Table 1. *Comparison of broth culture and microtest methods*

	Total no. of strains tested	Broth culture		Broth + cysteine culture H ₂ S production		Microtest + cysteine	
		+	–	+	–	+	–
<i>Salmonella</i> spp.	151	34	4*	112	1†	151	0
<i>Shigella</i> spp.	33	5‡	20	3	5	25	8
<i>Bact. aerogenes</i>	22	3	0	19	0	22	0
<i>Bact. cloacae</i>	3	1	0	2	0	3	0
<i>Bact. coli</i>	85	4	5	75§	1	85	0
<i>Bact. intermedium</i>	43	1	1	40	1	43	0
<i>Bact. pneumoniae</i>	18	3	3	9	3	18	0
<i>Bact. rhinoscleromatis</i>	5	0	1	2	2	3	2
<i>Bact. alcaligenes</i>	9	0	2	3	4	3	6
Paracolon group	36	11	5	19	1	34	2
<i>Proteus</i> spp.	44	0	0	44	0	44	0
<i>Pseudomonas</i> spp.	4	1	2	1	0	3¶	1
<i>Chromobacterium</i> spp.	9	3	0	4	2	9	0

* *Sal. paratyphi* A 3 strains, *Salm. typhisuis* 1 strain.

† *Sal. paratyphi* A 1 strain.

‡ Many of the + results with *Shigella* spp. were slow in both tests.

§ Two cultures variable on broth without cysteine.

|| Three weak positives.

¶ Weak positive.

Results are given either on broth or on broth + cysteine.

Broth tests read up to 7 days. Microtests read up to 24 hr.

Comparison of broth and microtest methods. Table 1 gives the results of microtest and broth culture methods. For cultures tested on both media only the broth + cysteine result is included. It will be seen that few cultures failed to produce H₂S from cysteine—some shigellas, alcaligenes, and paracolons. The *Salmonella* species negative on broth, including *Sal. paratyphi* A and *Sal. choleraesuis*, gave strong H₂S reactions in less than an hour in the microtest. *Sal. choleraesuis*, NCTC 5735, did not produce H₂S from broth or from broth + 0.01 % methionine, but gave a weak positive reaction from broth + 0.01 % cysteine in 24 hr. This culture was also tested on lead acetate agar containing 0.1 % cysteine with a lead acetate paper above (Table 2).

There was no blackening of the agar in 24 hr. in this test, but the lead acetate paper above turned black. This was an interesting indication of the greater sensitivity of the paper method.

Table 2. Comparison of methods for detecting H_2S production

Method	NCTC 5737 <i>Sal. choleraesuis</i> var. <i>kunzendorf</i>	
	NCTC 5735 <i>Sal. choleraesuis</i>	
	Result after 24 hr.	
1. Lead acetate agar	—	+
2. Lead acetate agar + 0.1 % cysteine	—	+
3. Lead acetate paper over medium 2	+	+
4. Lead acetate paper over Lemco broth	—	+
5. Lead acetate paper over Lemco broth + 0.01 % cysteine	+	+
6. Lead acetate paper over Lemco broth + 0.01 % methionine	—	+
7. Microtest with cysteine	+	+

Production of H_2S from sodium thiosulphate

Sodium thiosulphate is attacked by many bacteria with the production of H_2S . Microtests for H_2S production from sodium thiosulphate using 1 % $Na_2S_2O_3$ at pH 6.8 were run in parallel with the cysteine microtests (Table 3). The reaction was rather slower than with cysteine, blackening being first observed after 2–4 hr. Some of the negative strains were retested at pH 7.5 or 7.9 and gave slight blackening between 4 and 24 hr. In the routine test most of the strains were positive, though the reaction was usually weaker than with cysteine. The negative *Salmonella* species were *Sal. gallinarum*, *Sal.*

Table 3. Comparison of H_2S production from cysteine and thiosulphate

0.04 ml. 0.1 % cysteine hydrochloride or 1.0 % sodium thiosulphate, 0.04 ml. 0.25 M-phosphate buffer pH 6.8 and 0.04 ml. suspension. Tests read up to 24 hr.

	Total no. of strains tested	Microtest with cysteine		Microtest with thiosulphate	
		+	—	+	—
<i>Salmonella</i> spp.	60	60	0	52	8*
<i>Shigella</i> spp.	19	18	1	6	13†
<i>Bact. aerogenes</i>	7	7	0	7	0
<i>Bact. cloacae</i>	2	2	0	1	1
<i>Bact. coli</i>	77	77	0	63	14
<i>Bact. intermedium</i>	3	3	0	2	1
Paracolon group	18	18	0	15	3
<i>Pr. mirabilis</i>	16	16	0	15	1
<i>Pr. morganii</i>	19	19	0	13‡	6
<i>Pr. rettgeri</i>	6	6	0	0	6
<i>Pseudomonas</i> spp.	3	2	1	0	3
<i>Chromobacterium</i> spp.	6	6	0	5§	1

* Two strains retested at pH 7.5 positive.

† Seven strains gave weak positive reactions 24 hr. at pH 7.5.

‡ Three weak positives.

§ Two weak positives.

lexington, *Sal. paratyphi* A, *Sal. potsdam*, *Sal. pullorum*, and one strain each of *Sal. typhi*, *Sal. paratyphi* B and *Sal. typhisuis*. The two last were positive when retested at pH 7.5. Seven of the thirteen negative *Shigella* strains were weakly positive on retesting at pH 7.5. Of the *Proteus* strains none of the *Pr. rettgeri* produced H_2S from thiosulphate at either pH 6.8 or pH 7.5. Negative results with thiosulphate may only mean that the limit of sensitivity of this test has been reached, and not that the organism is incapable of attacking thiosulphate.

Production of H_2S from sodium sulphite

Sodium sulphite was also tested in a concentration of 1% at pH 6.8 or 7.5; few organisms produced more than traces of blackening in these tests, and it was not included in the routine series. The reaction was slower than with cysteine or thiosulphate, and blackening was seldom observed before 4 hr. and not until 24 hr. with most positive strains. The addition of glucose (Wilson, 1922-3) either to the medium on which the organism was grown or to the substrate made no difference to the results. Table 4 gives the results for the strains tested on cysteine, thiosulphate and sulphite. Strains negative with thiosulphate were never positive with sulphite.

Table 4. *Comparison of H_2S production from cysteine, thiosulphate and sulphite*

0.04 ml. 0.1% cysteine hydrochloride or 1.0% sodium thiosulphate or 1% sodium sulphite, 0.04 ml. 0.25M-phosphate buffer pH 6.8 and 0.04 ml. suspension. Tests read up to 24 hr.

	Total no. of strains tested	Microtest with cysteine		Microtest with thiosulphate		Microtest with sulphite	
		+	-	+	-	+*	-
<i>Salmonella</i> spp.	12	12	0	10†	2	5	7
<i>Shigella</i> spp.	11	11	0	7‡	4	4§	7
<i>Bact. coli</i>	7	7	0	6	1	2	5
<i>Proteus</i> spp.	9	9	0	5	4	0	9
<i>Pseudomonas</i> spp.	3	2	1	0	3	0	3
<i>Chromobacterium</i> spp.	6	6	0	5	1	3	3

* Most of the positive reactions were weak.

† Two variable.

‡ Six variable.

§ Two variable.

|| All *Pr. rettgeri*.

pH effect. There appears to be no well-defined pH optimum for these reactions under the conditions of test described above. Quantitative experiments were not carried out, but a rough estimate of activity was made by assigning arbitrary values to the degree of blackening of the lead acetate papers. *Bact. coli*, *Pr. morgani*i, *Sal. choleraesuis* and *Sal. choleraesuis* var. *kunzendorf* were tested at pH values between 4.2 and 9.0, with the results shown in Table 5.

Adaptation. Tarr (1933b) showed that the amount of enzyme in the washed suspensions was increased when cysteine was added to the medium on which the

cultures were grown, and that before H_2S was produced there was a lag period which could be eliminated by leaving the cells in contact with cysteine for some time or by growing the cells on cysteine reinforced agar. Desnuelle & Fromageot (1939) observed the same effect with *Bact. coli* and also found that the lag period was decreased in the presence of glucose. This effect was not directly investigated, but experiments were carried out to discover the effect on the microtests of suspensions from cultures grown on nutrient agar, glucose agar, and nutrient agar containing the test substrate. The nutrient agar used must have served as an adequate cysteine source for enzyme formation, as the activity of the suspensions from cysteine-reinforced agar was not increased. Most organisms produced blackening in 15–30 min. in the microtest. Growth on glucose agar or the addition of glucose to the reaction mixture made no appreciable difference.

Table 5. *Effect of pH on H_2S production in microtest*

Numbers = arbitrary values assigned to degree of blackening of lead acetate paper at 2 hr. Numbers in brackets = 24 hr. reading where 2 hr. reading was negative.

Culture	Substrate	pH values										
		4.2	4.7	5.0	5.6	6.2	6.8	7.2	7.8	8.1	8.5	9.0
		degree of blackening										
<i>Bact. coli</i> , NCTC 86	Cysteine	3	3	4	4	5	4	4	4	5	5	4
	$Na_2S_2O_3$	—	1	2	2	2	2	2	2	1	1	—
	Na_2SO_3	—	—	—	—	—	—	1	2	—	—	—
<i>Pr. morganii</i> NCTC 235	Cysteine	1	3	4	6	6	6	6	6	6	6	6
	$Na_2S_2O_3$	—	—	1	1	—	—	—	—	—	—	—
	Na_2SO_3	—	—	—	—	—	—	—	—	—	—	—
<i>Sal. cholerae</i> - <i>suis</i> , NCTC 5735	Cysteine	—	—	—	1	2	2	3	3	2	1	1
	$Na_2S_2O_3$	(2)	(2)	(2)	(2)	(2)	(2)	—	—	—	—	—
	Na_2SO_3	—	—	—	—	—	—	—	—	—	—	—
<i>Sal. cholerae</i> - <i>suis</i> var. <i>kunzensdorf</i> , NCTC 5737	Cysteine	1	1	2	2	3	3	4	2	2	1	1
	$Na_2S_2O_3$	(3)	(3)	(3)	(4)	(4)	(4)	(4)	(3)	(2)	(3)	(3)
	Na_2SO_3	—	(2)	(1)	(1)	(1)	(1)	(1)	(2)	(1)	—	—

Organisms grown on thiosulphate or sulphite agar showed little difference from control suspensions in their ability to attack these substrates, although this might have been shown if the H_2S had been estimated quantitatively. Occasionally suspensions from the reinforced agars appeared to give slightly faster and deeper blackening, but the effect was not consistent or very marked. One strain of each of the following organisms, *Bact. coli*, *Chr. prodigiosum*, *Pr. morganii*, *Pr. mirabilis*, *Sal. typhi*, *Sal. paratyphi* B, *Sh. boydii* and *Sh. flexneri*, was grown on nutrient agar, thiosulphate agar, and sulphite agar. Table 6 gives a typical result with *Bact. coli* on the three substrates.

DISCUSSION

It is clear from the conflicting results in the literature that the classification of organisms by their ability to produce H_2S offers many pitfalls. The results depend on the content and availability of the sulphur source, the sensitivity of the method used to detect H_2S , the growth of the organism on the medium,

Table 6. *The production of H₂S by Bacterium coli, NCTC 86*

Suspensions prepared from	Microtest with cysteine			Microtest with thiosulphate			Microtest with sulphite		
	Time of reading (hr.)								
	$\frac{1}{2}$	2	24	$\frac{1}{2}$	2	24	$\frac{1}{2}$	2	24
	Relative degree of blackening of lead acetate paper								
Nutrient agar	3	6	6	1	4	6	0	0	1
5% thiosulphate agar	1	4	6	0	1	4	0	1	2
5% sulphite agar	1	5	6	0	1	2	0	1	3

as well as the ability of the organism to elaborate H₂S-producing enzymes. Organisms such as *Proteus* which readily produce H₂S from organic media will be classified as H₂S-positive with any organic medium, whereas *Bact. coli* will be classified as positive or negative according to the test used (Hunter & Weiss, 1938). If we assume that cystine and cysteine are the main sulphur sources in peptone media, then positive and negative results for H₂S production on organic media might be a simple method of testing the presence or absence of a specific enzyme—cysteinase. This is apparently not the case. As early as 1912 Sasaki & Otsuka found that a wide range of bacteria all produced H₂S from an inorganic salt medium containing cystine, whereas not all of them produced H₂S on organic media. Other authors found that the addition of cystine or cysteine to their medium made their 'H₂S-negative organisms' appear positive. In this paper it has been shown that using non-multiplying suspensions with cysteine, very few of the organisms tested failed to produce enough H₂S to be detected by this test; positives included such species as *Sal. paratyphi* A, which is usually negative on organic media. There is, however, a quantitative variation; *Shigella* species were much less active and included cysteine-negative organisms which may, however, have produced amounts of H₂S below the limits of the method. With thiosulphate as a substrate the reaction appears to have rather more value in classification in that fewer organisms give positive results. Again the results are not clear-cut, and in the microtest the shigellas in particular offer an example of organisms so feebly active that they may appear positive or negative. Sulphite was not a suitable substrate for this technique, but the limited number of results obtained showed that it is less readily attacked by bacteria than either cysteine or thiosulphate.

The metal salts used as indicators of H₂S production in culture media not only vary in sensitivity but also in toxicity. Utermohlen & Georgi (1940), using a cysteine medium, found that nearly all the organisms tested tolerated 0.0001M-Co and 0.0005M-Ni as indicators. This medium was very sensitive and gave a large number of positives, but *Sal. typhi* was negative, suggesting that certain of its enzyme systems were particularly sensitive to cobalt and nickel. ZoBell & Feltham (1934) state that in classifying the *Brucella* group by production of H₂S on lead acetate agar there was so much difference in the

lead toleration of different brucellas that the test became one for lead toleration rather than H_2S production. Using a Bactotryptone without added sulphur source they found that newly isolated sewage organisms were less tolerant of metallic ions than laboratory stock strains, and that 0.2 mm-Pb, the lower limit for H_2S detection in their medium, retarded the growth of some sewage bacteria and that many more bacteria were partially suppressed by 0.5 mm-Pb. Iron gave a wider margin between indicating and toxic levels but was less sensitive at acid pH. Lead acetate papers were from 10 to 100 times more sensitive than the iron, lead or bismuth media, which ZoBell & Feltham (1934) tested. The bismuth medium developed by Hunter & Crecelius (1938) approaches the sensitivity of the lead acetate paper test.

The various selective and differential media based on testing for H_2S production are of undoubted value for the isolation and rapid investigation of bacteria of pathological significance. The method of choice will depend on the particular investigations, and the reports should indicate the medium used. For the complete description of enzymic capacity it is clearly necessary to give details of the sulphur source and the indicator, and all statements about H_2S producing capacity of an organism are incomplete unless the conditions of test are stated.

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(Received 30 October 1952)

'Degraded Vi Strains' and Variation in Vi-phage II of *Salmonella typhi*

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SUMMARY: The propagation of Vi-phage II on 'degraded Vi strains' of *Salmonella typhi* did not yield phage preparations bearing any trace of the specificity of the original Vi type from which the degraded strains had sprung.

Ten different standard Vi-typing phages were grown on a selection of degraded Vi strains. The phages could be separated into two groups: six underwent a change which appeared to be a phenotypic modification, and four were propagated unchanged. It seems probable, however, that all adapted Vi-typing preparations are phenotypic modifications of Vi-phage II.

In the attack of Vi-phage II on the typhoid bacillus the Vi antigen apparently plays a part only in the adsorption of the phage. The stages of phage growth succeeding adsorption seem to be independent of the Vi antigen, and it is in the regions of the bacterial cell concerned with these stages that Vi-type specificity seems to reside.

The typing of strains of *Salmonella typhi* by means of adapted Vi bacteriophages, first introduced by Craigie & Yen in 1938, has established itself as the most reliable guide in the epidemiological link-up of cases, carriers and vehicles of infection in typhoid fever. The standardized technique recommended by Craigie & Felix (1947) has been adopted as the provisional international standard method. It is based on the employment of standard Vi-phage preparations and the corresponding Vi-type strains that are distributed by the International Reference Laboratory for enteric phage typing (London). The revised typing scheme of *Sal. typhi* suggested by Craigie & Felix (1947) contained twenty-four types and subtypes. Five new types were added by workers in different countries during the next three years (International Committee for Enteric Phage Typing, 1950), and the extended scheme now in use, which was published in full in a preceding paper (Felix & Anderson, 1951a), comprises twenty-nine recognized types and subtypes.

Since the early work of Craigie & Yen (1938) it has been recognized that the chief difficulties encountered in the Vi-phage typing of typhoid strains are those arising from the 'degradation' of cultures. Various aspects of this phenomenon were subsequently discussed by Craigie (1942) and by Craigie & Felix (1947). More recently Felix & Anderson (1951a) described a striking instance of this complication met with in routine phage-typing.

The experiments recorded in the present paper were designed primarily to determine whether it would be possible to trace the specific Vi type from which a 'degraded' variant had arisen. It was hoped that the experiments might also throw some light on the mechanisms involved in the degradation of cultures of the specific Vi types of *Sal. typhi* and in the adaptation of

Craigie & Yen's Vi-phage II to the various Vi types. A preliminary note on the latter subject has already been published (Anderson & Felix, 1952).

'Untypable Vi strains' and 'degraded Vi strains'. A strain of *Sal. typhi* which contains the full complement of Vi antigen may be untypable for a number of reasons. First, it may be insensitive to Vi-phage II, either because it belongs to a type for which a specifically adapted phage has not yet been prepared, or because it is totally resistant to the action of Vi-phage II. The term 'untypable Vi strain' is now reserved for such strains and is no longer applied to all Vi-positive cultures which cannot be typed with Vi-phage II (see Craigie & Felix, 1947; Felix & Anderson, 1951*a*). Secondly, the strain may be a 'degraded Vi strain'. Degradation consists of the appearance of cross-reactions with heterologous typing phages so that the original specificity of the strain becomes obscured. In its most advanced stage the change results in the conversion of a specific type into Type A, the latter giving confluent lysis with all the typing phages. Tests of single colonies picked from a plating of a degraded culture may show that some colonies still give the specific phage reactions of the original type while others give the degraded reaction. Degraded colonies picked in this way may vary amongst themselves in the degree of cross-reactions. It is apparent then that the process of degradation may take place in a series of progressive stages of which the specific type occupies one extreme and Type A the other.

According to Felix (1951), only 3.0% of strains of the typhoid bacillus examined in the British Isles between 1942 and 1949 were 'degraded Vi strains' at the time the cultures were first subjected to the bacteriophage tests.

Craigie & Yen (1938) suggested that it might be possible by the adaptation of Vi-phage II to degraded variants of *Sal. typhi* to trace the specific types from which the variants had sprung. They called this method 'indirect typing'.

EXPERIMENTAL METHODS

Media

Tryptic digest broth was used throughout as the nutrient basis for media. When used as broth for the propagation of phages, for the growth of organisms alone, and for the preparation of dilutions of phages, 1% peptone was incorporated. The solid medium employed was tryptic digest broth with the addition of 1.3% New Zealand powdered agar but without peptone.

Preparation and testing of adapted phages

The standard typing phages used were titrated on the various strains selected for test. The plates were incubated overnight at 38.5°, and single plaques with a little of the surrounding culture were cut from those plates which showed discrete plaques, transferred to tubes containing 3 ml. of tryptic digest broth and incubated at 38.5° for as long as lysis continued. The average period of growth was 7 hr. When no apparent lysis took place and the turbidity of the tubes containing phage and culture increased at the same rate as that of the control tube containing culture without phage, incubation

for the full 7 hr. often resulted in loss of titre in the phages. In such cases the optimal incubation time for the production of phages of reasonably high titre was between 5 and 6 hr.

The newly grown phages were heated at 57° for 40 min. to kill the typhoid bacilli, centrifuged, and the supernatant fluid was pipetted off. Each phage preparation so obtained was titrated on the strain on which it had been grown and on the original Type A strain of Craigie & Yen (1938). This titration enabled suitable dilutions to be selected for test on all the Vi-type strains of the typhoid bacillus, in order to identify the type specificity of the new phages.

Table 1. *Example of preliminary titration of phage preparations*

Culture	Phage dilutions	Phage preparations grown from single plaques Phage E1/strain 9		
		Plaque 1	Plaque 2	Plaque 3
Type A	10 ⁻⁴	CL	CL	CL
	10 ⁻⁵	++n	+++n	CL
	10 ⁻⁶	+n	+n	++n
	10 ⁻⁷	1n	±n	+n
	10 ⁻⁸	1n	—	—
Degraded strain 9 (derived from Type D5)	10 ⁻⁴	CL	CL	CL
	10 ⁻⁵	+n	+++n	CL
	10 ⁻⁶	±n	+n	++n
	10 ⁻⁷	—	±n	+n
	10 ⁻⁸	—	1n	—
<i>Sal. typhi</i> , strain O901	10 ⁻²	—	—	—
<i>Sal. typhimurium</i> , strain 3040	10 ⁻²	—	—	—

CL=confluent lysis with standard loopful of test dilution of phage; —=no plaques; ±=5–10 plaques; +, ++, +++=increasingly numerous plaques; 1n=one normal plaque (diameter 1 mm. or more).

Table 1 gives an example of the preliminary titration of three phage preparations grown from selected single plaques. The table shows that each phage preparation was active to about the same titre on Type A and on the strain on which it had been propagated (degraded strain 9). In addition, two control strains were included in these tests as a routine procedure—*Sal. typhi* strain O901 and *Sal. typhimurium* strain 3040—in order to detect any contaminant O phages that might have been picked up accidentally during the process of propagation. These two strains, known from earlier work of one of us (A.F.) to be permanent Vi-negative variants, have been employed as reagents for the detection of O phages throughout the work on paratyphoid B Vi-phage typing (Felix & Callow, 1943, 1951). In the course of the present investigation it was found that the newly grown phage preparations invariably contained only the pure Vi-phage in the dilutions tested.

Phages were designated as fractions of which the numerator indicated the parent phage and the denominator the strain on which the phage had been propagated. For example, phage E1/strain 9 indicated a phage resulting from the propagation of the standard typing phage E1 on strain 9 (see Table 1).

If the preliminary titration showed that the propagated phages were of reasonably high titre, i.e. giving discrete plaques at a dilution of 10^{-7} to 10^{-8} on both Type A and the strain used for propagation, a full test was set up of selected dilutions of the preparation against the complete array of Vi-type strains of the typhoid bacillus (see Table 2). The dilutions used in these tests were: the highest dilution that produced confluent lysis on the propagating strain and on Type A, and a concentration one hundred times as strong as this. In this way the attack spectra of the newly propagated phages were determined, and their Vi-types identified.

Table 2. *Example of cross-tests for type specificity of phage preparations*

Vi-type strains	Dilutions of phage preparations grown from single plaques Phage E1/strain 9							
	Plaque 1		Plaque 2		Plaque 3		Control parent Phage E1	
	10^{-3}	10^{-4}	10^{-3}	10^{-4}	10^{-3}	10^{-4}	10^{-3}	0.8×10^{-4}
	CL	SCL	CL	CL	CL	CL	CL	SCL
A	CL	SCL	CL	CL	CL	CL	CL	SCL
C	$\pm m$	—	$+++m$	$1m$	CL	$7m$	$+s$	—
D1	$4m$	—	$\pm m$	—	$+m$	—	$\pm m$	—
D2	—	—	$2m$	—	—	—	—	—
D4	$8m$	—	—	—	$5m$	—	$2m$	—
D5	—	—	$\pm m$	—	$\pm m$	—	$5m$	—
D6	—	—	—	—	—	—	—	—
E1	$2m$	—	$\pm m$	—	$+m$	$2m$	CL	SCL
E2	—	—	—	—	—	—	$4n$	—
F1	—	—	—	—	—	—	—	—
F2	—	—	—	—	—	—	—	—
G	$1m$	—	$2m$	—	$\pm s$	—	—	—
H	$+m$	—	$+\pm n$	—	$++++m$	—	$\pm m$	—
J	—	—	$1m$	—	$2m$	—	—	—
K	—	—	—	—	—	—	—	—
L1	—	—	$2m$	—	$2m$	—	—	—
L2	—	—	$1m$	—	$\pm m$	—	—	—
M	—	—	$\pm m$	—	$3m$	—	—	—

SCL=semi-confluent lysis; s =small plaques, visible to the naked eye; m =minute plaques, visible only by means of magnifying lens ($\times 10$). Other symbols as Table 1.

Table 2 illustrates in abridged form the cross-tests for type specificity of phage preparations. In the experiment recorded in Table 2 none of the three single-plaque phages tested gave the same lytic reactions as the parent phage E1, but all reacted in a way identical with that of the standard typing phage A. The latter has been omitted from the table for the sake of simplicity.

Phage preparations grown from 149 single plaques were examined in the manner illustrated in Tables 1 and 2. The experiments were divided into two groups.

RESULTS

Experiments in group 1

In this group of experiments phage A was propagated on a number of Type A strains encountered in the routine work of the laboratory. The strains had been isolated from outbreaks occurring in this country at different times and in

different localities. Type A is the most prevalent of the typhoid Vi-phage types indigenous to Great Britain; its average percentage distribution during the eight years 1942-9 was 27.3 % (Felix, 1951). As it is believed that many of these strains are descended from specific Vi-phage types which have undergone degradation, these experiments were carried out in order to determine whether any residuum of specificity remained in the strains that could be transmitted to the phages grown on them.

This series can be summarized briefly. All phages resulting from the propagation of phage A on Type A strains were unchanged phage A. From this two possible conclusions could be drawn. Either the Type A strains employed were not descended from specific types or, if they were, the transition to Type A was accompanied by a loss of the ability to produce phages bearing any specificity for the original type.

Experiments in group 2

In the second group of experiments ten of the standard typing phages were grown on degraded variants of strains which originally had been found to belong to some of the recognized Vi-phage types. The following phages were used in this series: phages C, D4, D5, D6, E1, F1, L1, N, O and T. Table 3 lists the eight strains of *Sal. typhi* employed and also shows which phages were propagated on each strain.

Table 3. *List of 'degraded Vi strains' and specific Vi-type phages used in the experiments in group 2*

Degraded Vi strains			
Original Vi-phage type of strain	Serial no. of strain	Vi-phage reaction of selected 'degraded' substrains	Standard typing phages propagated on the degraded substrains
D5	9	Type A	C, D4, D5, D6, E1, F1, L1, N, O, T
D5	10	Type A	D4, D6, F1, L1, N, T
N	1	Degraded	D6, E1
N	2	Degraded	D5, T
N	3	Degraded	F1, O, N
O	7	Type A	C, D4, D5, D6, E1, F1, L1, N, O, T
O	8	Type A	C, D4, D6, F1, L1, N, T
T	11	Type A	C, D4, D5, D6, E1, F1, N, O, T

Table 3 shows that all the strains had lost the specific Vi-phage reactions of the strains from which they sprang. Those derived from the three Type N strains (nos. 1, 2 and 3) gave confluent lysis with phages N and T; semi-confluent lysis or less with phages D1, D2, D4, D6, F1 and G; and cross-reactions of varying degree with the other typing phages. All colonies of the former Type N strains exhibited this partly degraded reaction. The remaining five strains gave the full reaction of Type A.

The strains were plated and single colonies picked and tested with the standard typing phages. Though most of the single colonies of strains which reacted as Type A were fully susceptible to all the typing phages, some showed the less complete degraded reaction. Only lines giving the full cross-reaction

of Type A were used in subsequent experiments on these cultures. Single-colony lines were retained for all the experiments in group 2, and the purity of these lines was checked daily by careful colony selection.

In the preliminary titration of the adapted phages used as the starting points of this series, it was found that their titres on each of the degraded cultures giving the Type A reaction were the same as those on the homologous strains for which they were specific: for example, phage E1 titrated on strain 9 and on Type E1 gave individual plaques at about 10^{-8} on both strains.

The ten specific *Vi*-typing phages grown on degraded organisms giving the full Type A reaction could be separated into two groups as shown in Table 4. Four of the phages were propagated unchanged and five were changed to phage A. One of the phages (D4) is not included in Table 4. Phage D4 was propagated as phage D1 on Type A; this will be discussed later.

Table 4. *Results of propagation of specific Vi-type phages on Type A cultures derived by degradation from specific Vi-type strains*

Phages changed to phage A on propagation	Phages propagated unchanged
C	D5
E1	D6
F1	O
L1	N
T	

The change from the various adapted phages to phage A occurred uniformly in all of the many plaques cut. The phage A so produced was indistinguishable from the ancestral phage A, being highly specific for Type A at selected concentrations but containing variant particles able to attack other types; no indication could be found that the phages had propagated in their former type-specific state.

In contrast, the four phages D5, D6, O and N were propagated unchanged, and we were unable to change them to phage A by propagation on organisms giving the full Type A reaction.

Cultures which gave the 'degraded' reaction, i.e. those descended from the three strains of *Vi*-phage Type N (see Table 3, strains 1, 2 and 3) were much more difficult to work with than were those reacting as Type A. In the first place the sensitivity of these strains to the *Vi*-typing phages used in this investigation was relatively low, and individual plaques were extremely small and difficult to find. As we considered it essential to work with pure-line phages this constituted a considerable obstacle to the work. Nevertheless, a few phages were grown by propagating phage T on one of the degraded Type N substrains. All the resultant phages conformed to the same pattern, that is, they attacked Types A, N and T with equal ease, a finding which indicated indeterminate specificity in these phages.

DISCUSSION

The experiments described in this paper show that it is not possible, by the adaptation of Vi-phage II to a degraded variant, to trace the specific type from which the degraded variant has sprung. This finding is of considerable practical importance and was reported at the Fifth International Congress for Microbiology in Rio de Janeiro (International Committee for Enteric Phage Typing, 1950). 'Degraded' Type A variants are occasionally isolated during the course of typhoid outbreaks (see Craigie & Felix, 1947; Felix & Anderson, 1951*a*). Their common origin with the specific type responsible for the outbreak cannot be established by Vi-phage typing, and it is therefore necessary to seek other criteria to prove this identity of source. Colonial morphology, nutritional requirements, fermentation reactions and sensitivity to phages unrelated to Vi-phage II were all found to be useful ancillary criteria (Felix & Anderson, 1951*a*). Epidemiological evidence may of course be of help, and workers in this field recognize the fact that the appearance of degraded variants in association with one of the specific Vi-phage types of *Sal. typhi* is occasionally, though fortunately rarely, to be expected.

The difficulty arising from the degradation process—the inability to connect the variant with its parent strain by the phage-typing method—can be reduced to some extent by making use of the fact that the variants occur in clones in platings of specific types showing the degradation. If, during the primary isolation of the typhoid bacillus, a number of colonies are pooled from platings to form the stock culture, the chances of finding the ancestral specific type as well as its degraded progeny are greatly increased. This is a point of considerable importance, as there is a tendency to send single-colony subcultures to reference laboratories for phage typing. If this procedure is followed after enrichment through tetrathionate or selenite broth the risk of a degraded variant being selected is materially increased. For the preparation of cultures submitted for phage typing the pooling of twelve selected colonies is recommended, preferably from primary platings on selective media such as deoxycholate citrate or Wilson Blair agar. Of course, this is not always possible, as very few colonies of the typhoid bacillus may grow in primary platings. On the other hand, in positive blood cultures, a number of colonies are always available for pooling. Twelve-colony subcultures enable a representative sample of the parent strain to be examined, and the possibility of the picture being complicated by degraded variants is thereby reduced. It may be mentioned also that not only may Vi degradation of the kind described be apparent in single-colony subcultures, but that there is an appreciable risk of selecting a Vi-negative variant which will resist the Vi phage altogether. This point has been emphasized in previous papers (Felix, 1948, 1951). If the organism can be isolated from the patient again a twelve-colony culture will usually reveal its Vi-phage type. But it is not always possible to isolate *Sal. typhi* from cases of typhoid fever more than once, and if later cultures cannot be obtained the phage type of the infecting strain may never be determined and information of epidemiological value is lost.

Degradation is associated with the loss of Vi-type specificity, but it is not necessarily associated with a loss of virulence, and Type A strains may show a degree of virulence approaching that of the classical strain Ty 2, which is maximal. An example of this was described recently (Felix & Anderson, 1951*a*).

Mechanism of degradation

The mechanism of degradation can be clarified to some extent by combining information afforded by the present experiments with that resulting from work on the phages carried by various Vi types of the typhoid bacillus. It has been shown (Craigie, 1946; Felix & Anderson, 1951*b*; Anderson, 1951) that Vi-phage type specificity is controlled in a number of types of *Sal. typhi* by the carriage of bacteriophages by the types concerned. Moreover, these type-determining phages are O and not Vi phages, as indicated by their lytic action for the Vi-negative strain O901 (Anderson, 1951; Anderson & Felix, 1953). Furthermore, the artificial production of new Vi-phage types in the laboratory by the treatment of susceptible types such as Type A with the type-determining phages may be accompanied by a change in susceptibility to O phages (Felix & Anderson, 1951*b*); and certain naturally occurring lysogenic Vi-phage types of the typhoid bacillus constantly resist one of the high-titre salmonella O phages described by Felix & Callow (1943) that has been in routine use in this laboratory for the past twelve years. These facts make it appear probable that the Vi antigen acts only as the primary receptor for the attachment of Vi-phage II to the organisms; but, having filled this role, it does not seem to play any further part in the determination of Vi-phage type specificity. This argument is supported by the observations of Craigie (1940) that any Vi-phage type of *Sal. typhi* will adsorb any adapted Vi-phage II preparation, homologous or not with the type, but will only be lysed if the phage type of the organism and the type specificity of the phage are the same.

These findings suggest that the attack of Vi-phage II on the typhoid bacillus can be divided into two stages. The first is the adsorption of the Vi phage to the Vi antigen; this stage is specific only in so far as it is dependent on the presence of the Vi antigen. The second stage, leading to multiplication of the phage, is highly specific and only occurs when the types of the phage and organism are the same. It is not known at what point of the cycle the second stage of attack is blocked when the types of phage and organism are different. However, in view of the fact that phage-type specificity is often dependent on lysogenicity, it seems probable that the block would be an exclusion phenomenon due to the presence in the bacterial cell of the type-determining phage.

Two further points merit consideration. First, it has been demonstrated (Felix & Anderson, 1951*b*; Anderson, 1951) that phage-free organisms appearing in cultures the Vi-type determination of which depends on lysogenicity acquire a greater range of susceptibility to the Vi-typing phages immediately the carried phage is lost. Secondly, the treatment of the fully sensitive Type A with type-determining phages produces specific Vi-types with a very much reduced range of susceptibility to the Vi-typing phage prepara-

tions (Craigie, 1946; Felix & Anderson, 1951*b*; Anderson, 1951; Anderson & Felix, 1953). In these cases, then, the width of the spectrum of sensitivity to Vi-typing phages is controlled by the presence or absence in the organism of certain O phages. Nicolle, Hamon & Edlinger (1951) and Hamon & Nicolle (1951) have published similar observations on *Sal. paratyphi* B.

For some time before the experiments recorded in this paper were begun (1947), one of us (A.F.) held the view that the process of degradation of specific Vi-phage types was an instance of variation by loss. It was assumed that every Vi-positive strain of *Sal. typhi*, irrespective of its Vi-phage type, possessed all the specific receptors corresponding to the known adapted Vi-type phages; that freshly isolated bacilli belonging to one of the specific Vi-phage types also possessed a full range of inhibitors which rendered inaccessible those receptors corresponding to the heterologous phages; that the inhibitors were gradually lost on subculture, thereby exposing more and more of the heterologous receptors, until finally all the inhibitors were lost and a culture of Type A emerged. The designation 'degraded Vi strain' thus implied that the process was an instance of variation by loss, but the concept was based on mere speculation. The recent work on type-determining phages, quoted in the preceding paragraphs, suggests that in some of the recognized Vi-phage types of *Sal. typhi* the change in the direction of Type A occurs in a single step, and is associated with the loss of a single factor, namely, the type-determining phage, which is an O phage. Although the process of type determination does not take place within the Vi-antigen complex, it appears nevertheless to be convenient and justifiable to continue to use the term 'degraded Vi strain', since the bacteria are Vi forms that have undergone loss variation.

Adaptation of Vi-phage II

It is now possible to examine the information afforded by the present experiments on the mechanism of adaptation of Vi-phage II to the various Vi-types of *Salm. typhi*. Throughout the following discussion the terms 'phenotypic variation' and 'host-range mutant' are used in order to interpret the phenomena observed in a way comparable to that in current use by bacteriophage workers.

Craigie & Yen (1938) suggested at an early stage in this work that the acquisition of type specificity by the adapted Vi-typing preparations was due to the selection by the different strains of *Sal. typhi* of host-range mutants already present in any phage preparation used for adaptation. Craigie (1940) gave estimates of the frequency of occurrence of host-range mutants corresponding to a number of specific Vi-types. Craigie & Yen used this concept as the working hypothesis in the selection of their different Vi-type strains of the typhoid bacillus, and it has served a most useful purpose. On the other hand, Felix (1949) found it difficult to accept this hypothesis on the ground that it necessitates the assumption that Vi-phage II and *Sal. typhi*, two apparently distinct biological entities, have evolved two exactly corresponding sets of mutants. He argued that the observed phenomena could be explained with less strain on the imagination on the basis that phage was a self-repro-

ducing unit of endogenous origin, forming part of the genetic make-up of the bacterium.

However, the experiments described in this paper show that the 'host-range mutant' conception is not entirely necessary for the explanation of *Vi*-phage II adaptation. Table 4 shows that, of ten adapted phages employed, five changed to phage A on propagation on Type A strains. It has been mentioned in the preceding section that, in the preliminary titration of the original adapted phage preparations, it was found that their titres on the homologous specific type strains were the same as those on the Type A cultures of the degraded strains on which it was intended to propagate the phages. From this it could be concluded that every particle of each phage preparation was equally active both on the homologous and the Type A strains. All plaques cut from titrations on Type A strains of each of the five phages in the left-hand column of Table 4, and propagated in the manner described earlier, yielded phage A. This newly grown phage A was indistinguishable from the ancestral phage A from which most of the standard typing phages are descended. As no indication could be found that the phage had propagated as the former type, it was concluded that the change from the former type (i.e. C, E1, F1, etc.) to phage A had occurred in a single step commencing with the particle initiating each plaque which had been cut. This 100 % reversion of adapted phages to phage A suggests that, whatever is responsible for the production by certain particles of adapted phages with a newly acquired host spectrum, the process is not one of selection of host-range mutants but of particles susceptible to a modification of host spectrum without genetic alteration. The change is maintained only as long as the adapted phages do not multiply or the bacterial host on which they are allowed to multiply belongs to the same *Vi*-phage type as that used for the last adaptation. On the other hand, propagation on Type A changes all particles to phage A in a single step. The adaptation may thus be described as a phenotypic variation.

It is not clear from these experiments whether the initiation of plaques during the adaptation of phage A to the various *Vi*-phage types of the typhoid bacillus results from the selection of particles possessing this phenotypic plasticity, or of organisms metabolically different from the general population that are able to initiate the change in any particle. The fact that the number of plaques produced by phage A on a heterologous type is proportional to the concentration of phage suggests that the former hypothesis is more likely to be correct.

In contrast to these findings are those with phages D5, D6, O and N (see Table 4) which propagated unchanged. In the course of many experiments with these phages grown on Type A cultures we were unable to change them to phage A, and it was provisionally concluded that they were host-range mutants with genetic continuity. That a phage behaving as a host-range mutant may nevertheless show phenotypic variation is apparent from the change of phage D4 to phage D1 on Type A strains. Phage D4 was first prepared by adapting phage A to Type D1 (Craigie & Yen, 1938) and then adapting the resulting phage D1 to Type D4 (Felix, 1943). Phage D1 could

thus be regarded as a selected host-range mutant of phage A and phage D4 as a phenotypic variation of phage D1.

Phages grown on organisms giving the partly degraded reaction were few in number because of technical difficulties which have been indicated earlier. The only useful phages in this group were derived from the propagation of phage T on degraded Type N strains. The phages so obtained, as has already been pointed out, could not be assigned to one of the recognized Vi-types because they showed lytic activities of equal strength for Type A, Type T and Type N. The interpretation of this lack of specificity was something of a problem until it was realized that if one considered phage T to be a phenotypic variation of Vi-phage II, contact of such a phage with a strain lacking the distinctive characters of one of the specific Vi-types might impress on it a further phenotypic variation, resulting in a phage that could not be identified by testing on the recognized specific Vi-types of *Sal. typhi*. This change, therefore, appears to be the same in principle as the others which have been interpreted as phenotypic variations, and it is interesting to note that phage T belongs to this group when tested on Type A cultures.

In spite of the fact that only six of the ten adapted phages used in these experiments changed their type when propagated on Type A cultures derived from degraded Vi strains, we are inclined to believe that the whole process of adaptation of Vi-phage II to the various Vi-types of the typhoid bacillus is one of phenotypic variation. It is possible that we were unsuccessful in changing the remaining four phages to phage A because the correct organisms were not employed to precipitate the change.

If it is assumed that the acquisition of Vi-type specificity by Vi-phage II is entirely phenotypic in origin, the difficulty of believing that Vi-phage II has independently evolved variants corresponding to all the possible Vi-types of *Sal. typhi* disappears. It is only necessary to postulate a labile grouping on the bacteriophage that can be modified, during the processes following adsorption, to fit the specific patterns in the various phage types of the organism. Type specificity is thus considered to be inherent in the bacterium only. This hypothesis is clearly applicable to those phages which changed to phage A, to the change of phage D4 to phage D1, and to the change of phage T to one of ill-defined specificity; whether it will be possible to prove that the remaining adapted Vi-phages belong essentially to the same category can only be decided by further work.

It is evident that the general rule, laid down by Craigie & Felix (1947), of using phage A as the parent phage for adaptation to new typhoid Vi-types in the preparation of the standard typing phages is a sound one. Phage A is highly specific for Type A, and a new phage descended from it acquires the additional ability to attack only the new type used for adaptation. In contrast, phages descended from a host-range mutant, such as phage D6, may retain in full the ability to attack Type D6 and can then lyse Type A, Type D6 and the type to which they have been newly adapted.

Interest has been aroused recently in the subject of phenotypic variation in phages, and Luria & Human (1952) and Bertani & Weigle (1953) have

published work which is in many respects similar to that described in this paper, although the phages and host organisms were different. The phenotypic variations impressed on Vi-phage II are directed, at least in some instances, by the alterations produced in the typhoid bacillus by apparently unrelated non-Vi-phages. Weigle & Delbrück (1951) demonstrated the exclusion of a carried phage (λ) in ultra-violet induced (Lwoff, Siminovitch & Kjeldgaard, 1950) *Bact. coli* K-12 by infection with phage T5. They argued that, as phage λ was already present in the bacterial cells exposed to attack by T5, it was probable that the exclusion took place at an intracellular level, and that exclusion by resistance of the bacterial cells to penetration as suggested by Lesley, French, Graham & Van Rooyen (1951*a, b*) could be ruled out. In the resistance of the lysogenic Vi-phage types of *Sal. typhi* to phage A the position is reversed; the carried phage excludes the externally applied phage, and it is evident that the block is at some stage in the multiplication cycle of the invading phage succeeding adsorption. Thus the altered physico-chemical patterns produced in the organism by lysogenicity are at the same time responsible for the resistance of the lysogenic types to phage A, and for the phenotypic modification of a fraction of the Vi-phage II particles into forms which are able to multiply in the bacterial cell; these forms constitute the range of Craigie's adapted Vi-phages.

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(Received 3 November 1952)

SINGH, B. N. & CRUMP, L. M. (1953). *J. gen. Microbiol.* 8, 421-426.

The Effect of Partial Sterilization by Steam and Formalin on the Numbers of Amoebae in Field Soil

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SUMMARY: In Sitka spruce nursery plots the numbers of amoebae in steam-treated soil rose with the increase in the bacterial population. The population of amoebae over a period of seven months was much higher in this soil than in the untreated or the formalin-treated soils. The formalin-treated soil had significantly lower numbers of amoebae compared with the untreated soil over a period of one year, although the bacterial numbers were often higher in the former than in the latter. It is suggested that the unsuitable quality of bacterial food supply might be responsible for keeping the numbers of amoebae in check in the formalin-treated soil. Double formalin treatment seemed to suppress further the numbers of amoebae.

The occurrence of Protozoa in soils was known to the biologists of the nineteenth century. Russell & Hutchinson's (1909) theory to explain the effects of partial sterilization stimulated the development of research on soil Protozoa. This theory drew attention to the fact that many soil Protozoa are primarily devourers of bacteria. It attempted to account for the development of 'soil sickness' by supposing this to be due to an excessive number of active Protozoa which diminished the bacterial population and for the remedial effect of partial sterilization by the suppression of these Protozoa by the sterilizing agents. The theory put forward by Russell & Hutchinson has never been fully accepted. Laboratory experiments with pure cultures of bacteria with and without the additions of Protozoa have shown that the latter do not always depress the biochemical activity of the bacteria, but may in fact stimulate it under certain conditions (Nasir, 1923; Cutler & Bal, 1926; Cutler & Crump, 1929; Harvey & Greaves, 1941; Meiklejohn, 1930, 1932). Further critical work is needed to explain this most interesting phenomenon.

Amoebae are numerically the most important group of soil Protozoa which feed on bacteria. Their importance is perhaps increased by the fact that they feed differentially (Singh, 1941, 1942, 1945, 1947*a, b*, 1948) and are known to diminish bacterial numbers in sterilized soil. It seems, therefore, that this group is of prime importance to the examination of the Protozoa theory of partial sterilization. The earlier work on amoebae in partially sterilized soils has been carried out under laboratory conditions. The programme of field trials carried out at Ampthill on Sitka spruce nursery plots, some of which were treated with steam and formalin, provided an opportunity to investigate the action of these agents on numbers of amoebae and bacteria under field conditions, while modern technique for estimating numbers of amoebae

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(Singh, 1946) has greatly improved the validity of results now obtainable. The present paper records the results of such estimates made from samples taken at intervals over a period of twelve months from the time of applying the sterilizing agents to the plots.

MATERIALS AND METHODS

The plots from which the samples were taken were part of an experiment conducted by the Chemistry Department of Rothamsted Experimental Station at the Ampthill Forest Nursery, Bedfordshire, on a light sandy soil having a pH value of about 6.0, which was not appreciably changed by the treatments. The main results of this experiment will be published elsewhere.

Small plots were steamed for about 20 min., using four grids at a time, each grid having five pipes, 4 ft. long and 9 in. apart buried at a depth of about 9 in. A solution of 10% commercial formalin in water at the rate of 1 gallon/sq.yd. was added to the formalin-treated plots. A more detailed description of the plots is given by Mollison (1953). The dates on which steam and formalin were applied to the soil and the intervals at which the amoebae were counted are given below.

Twelve 6 in. borings from one plot of each treatment were taken and thoroughly mixed to form a composite sample. The samples were brought to the laboratory and passed through a 3 mm. sieve. The numbers of amoebae were estimated by the dilution technique of Singh (1946), eight replicate subsamples being tested from each dilution. The cultures in which amoebae could be seen by microscopical examination were considered positive. The analysis of the data is based on the theory developed by Fisher (cf. Introduction to Fisher & Yates, 1947; Appendix, Singh, 1946). With the technique used a difference of approximately 100% in populations estimated from two individual samples is significant at 5% level.

No counts of amoebae from duplicate soil samples were made; the differences between a number of duplicate field soil samples in earlier work (Singh, 1946, 1949) were in no case found to be significant.

The bacterial numbers were estimated by plate counts on the following medium: $\text{Ca}(\text{NO}_3)_2$, 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g.; Ca gluconate, 0.2 g.; K_2HPO_4 , 0.1 g.; KH_2PO_4 , 0.1 g.; NaHCO_3 , 0.2 g.; H_2O , 1 l.; FeCl_3 , 0.2% (w/v.), 1 ml.; agar, 15 g.; filter and add mannitol, 0.1 g.; asparagine, 0.1 g.; 'Difco' yeast extract, 0.1 g., adjusted to pH 7.4.

The results given below were derived from three different field trials carried out at Ampthill in 1950, 1951 and 1952.

RESULTS

Experiment 1, 1950

Table 1 gives the counts of amoebae in untreated, formalin-treated and steam-treated soils. In the tables the numbers of active amoebae given are obtained by subtracting cystic from total numbers. Thus where these do not differ significantly the number of active forms may be nil. The soils were

Table 1. *The numbers of amoebae and bacteria in untreated, formalin-treated and steam-treated soils under natural conditions*

Date	Amoebae count (no./g. wet soil)			Bacterial count (10 ⁶ /g. wet soil)	Soil water content (%)
	Total	Cystic	Active		
23 Mar. 1950	12,200	979	11,221	3.3	12.4
20 Apr. 1950	8,570	3,800	5,270	10.75	12.6
4 May 1950	10,200	4,670	5,530	13.0	11.8
25 May 1950	15,800	6,040	9,760	—	—
8 June 1950	17,300	4,670	12,630	11.9	8.5
6 July 1950	14,500	3,600	10,900	10.4	11.2
31 July 1950	13,300	4,280	9,020	5.25	11.0
24 Oct. 1950	17,300	4,670	12,330	8.3	8.4
Formalin-treated soil					
23 Mar. 1950	685	412	—*	2.5	12.6
20 Apr. 1950	1,650	1,390	—*	27.55	12.1
4 May 1950	2,770	1,270	1,500	13.75	11.7
25 May 1950	11,100	5,540	5,560	—	—
8 June 1950	8,570	2,770	5,800	28.35	8.5
6 July 1950	7,210	3,020	4,190	14.15	8.6
31 July 1950	11,100	5,540	5,560	7.0	10.9
24 Oct. 1950	10,200	5,540	4,660	20.1	7.5
Steam-treated soil					
23 Mar. 1950	693	126	567	5.0	13.9
20 Apr. 1950	12,200	2,540	9,660	27.85	12.8
4 May 1950	20,500	5,540	14,960	38.25	13.9
25 May 1950	54,000	12,200	41,800	—	—
8 June 1950	37,900	1,960	35,940	42.55	8.8
6 July 1950	59,000	5,540	53,460	18.0	10.0
31 July 1950	45,200	5,540	39,660	8.3	13.1
24 Oct. 1950	45,200	13,300	31,900	15.65	8.6

* Total and cystic not significantly different.

treated with steam on 10 March and with formalin on 16 March. In the first count taken on 23 March the total numbers of amoebae found in steam- and formalin-treated plots (column 2) did not differ significantly from the number of encysted forms found in the untreated plot (column 3). This suggests that both treatments killed the active forms. At this date the amoebae in the formalin-treated soil were still nearly all in the cystic condition, since the numbers of encysted amoebae (column 3) did not differ significantly from the total numbers (column 2). In the case of steam-treated soil the majority of the amoebae were beginning to excyst, most of them being in the active condition (column 4). The next count taken on 20 April showed that the population of amoebae had greatly increased in both the formalin- and steam-treated soils. In subsequent samples the amoebae in the steam-treated soil were several times higher than the control soil, and a very large proportion of them were in the active condition. This clearly demonstrates that the beneficial effect of partial sterilization by steam is not due to the killing of the amoebae.

The bacterial population in the steamed soil was very much higher than the control. This treatment probably stimulates the growth and the reproduction of amoebae owing to the increase in the amount of bacterial food. The numbers of amoebae were very much lower on the whole in the formalin-treated than in the untreated plot, although the bacterial numbers were, after the first 4 weeks, higher in the former soil. Thus it seems that some factor or factors other than bacterial numbers were responsible for keeping the numbers of amoebae in check in the formalin-treated soil.

Experiment 2, 1951

Table 2 gives the results of samplings from a second experiment. The soil was treated with formalin on 13 March and the first count was taken on 8 May. In this and in all later counts taken over a period of 1 year the total

Table 2. *The numbers of amoebae and bacteria in untreated and formalin-treated soils under natural conditions*

Date	Amoebae count (no./g. wet soil)			Bacterial count (10 ⁶ /g. wet soil)	Soil water content (%)
	Total	Cystic	Active		
Untreated soil					
8 May 1951	10,200	2,330	7,870	12.4	12.7
5 Feb. 1952	13,200	5,540	7,660	9.2	—
3 Apr. 1952	11,100	5,540	5,560	19.65	—
13 May 1952	15,800	3,330	12,470	7.8	13.4
17 June 1952	10,200	3,600	6,600	9.75	10.0
Formalin-treated soil					
8 May 1951	534	377	—*	42.85	11.4
5 Feb. 1952	2,540	490	2,050	8.05	—
3 Apr. 1952	1,960	639	1,321	32.2	—
13 May 1952	3,960	534	3,426	7.45	12.9
17 June 1952	3,600	1,270	2,330	8.75	10.0

* Total and cystic not significantly different.

numbers of amoebae were significantly lower in the formalin-treated than the untreated soil. The amoebae were present in active and cystic condition in both soils. This observation confirms the findings of the previous year.

Experiment 3, 1952

Table 3 shows the results of a few counts of amoebae and bacteria in untreated, formalin-treated and twice formalin-treated soils. The formalin was applied to the soil on 29 February. The soil termed 'twice formalin-treated' was from the plot (Exp. 2) that received formalin in 1951 and was given a second dose in 1952. Although the counts of amoebae were not continued for a long period, the results confirm those of previous years and also suggest that the effect of two treatments with formalin further decreased the population of amoebae.

Table 3. *The numbers of amoebae and bacteria in untreated, formalin-treated and twice formalin-treated soils under natural conditions*

Date	Amoebae count (no./g. wet soil)			Bacterial count (10 ⁶ /g. wet soil)	Soil water content (%)
	Total	Cystic	Active		
3 Apr. 1952	11,100	5,540	5,560	19.65	—
13 May 1952	15,800	3,300	12,470	7.8	13.4
17 June 1952	10,200	3,600	6,600	9.75	10.0
Formalin-treated soil					
3 Apr. 1952	582	979	—*	41.45	—
13 May 1952	1,800	582	1,218	21.5	13.3
17 June 1952	5,080	2,140	2,940	9.05	10.4
Twice formalin-treated soil					
3 Apr. 1952	265	154	—*	34.9	—
13 May 1952	1,270	265	1,005	22.85	13.8
17 June 1952	1,800	635	1,165	8.0	10.7

* Total and cystic not significantly different.

DISCUSSION

According to Russell & Hutchinson's (1909) theory of partial sterilization the treatment of soil by steam or various volatile chemical antiseptics has a detrimental effect on the Protozoa which were thought to be the agents in causing 'soil sickness'. The counts of amoebae in this paper clearly demonstrate that no generalizations can be made as to the effects of partial sterilizing agents on the subsequent growth and multiplication of amoebae in field soils. In the case of steam treatment the population of amoebae is ultimately much higher than in the untreated soil. Formalin treatment considerably lowered the numbers of amoebae, but the bacterial numbers were often higher than in the untreated soil in all the experiments. It may be that the factor responsible for keeping the amoebae in check is the unsuitable type of food bacteria which develop in formalin-treated soil. One of the authors (L.M.C.) has found that the bacterial flora which develop in steam- and formalin-treated soils are to some extent qualitatively different, and that this difference persists over a long period. Further work on the bacterial relationships in soils partially sterilized by volatile antiseptics is needed to investigate the influence of partial sterilization on the feeding qualities of the different bacterial populations. The importance of the quality of the bacterial flora for the growth of amoebae in field soils was also suggested by the different effects of artificial fertilizers and dung on the numbers of amoebae in Rothamsted soils (Singh, 1949). The results obtained from these Rothamsted plots as well as those here recorded from partially sterilized soils suggest that the quality of the bacterial flora in the field is of more importance than its quantity in determining the numbers of amoebae, a result to be expected from laboratory experiments on the differential feeding of these organisms (Singh, 1941, 1942, 1945, 1947*a*, *b*, 1948).

It is a pleasure to express our thanks to Dr H. G. Thornton, F.R.S., for his interest in this work. Our thanks are also due to Miss Mabel Dunkley for her assistance in connexion with the preparation of the manuscript.

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(Received 6 November 1952)

CARR, J. G. (1953). *J. gen. Microbiol.* 8, 427-433.

The Association of Haemin with the Fowl Cancer Viruses

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SUMMARY: Haemin was found in all specimens of purified preparations of Rous I, Duran-Reynals B, D and V, Fujinami, and MH2 viruses, and also in macromolecular preparations from non-filterable fowl sarcomas and normal fowl tissues. The amounts were not constant in different batches of Rous I, and it was shown that this virus would strongly adsorb pure haemin from solution. It is suggested that most, probably all, of the haemin encountered in these preparations was derived from the host blood. Adsorbed haemin had no effect on the oxidative destruction of the virus, but slightly altered its physical properties.

It was noticed that preparations of fowl sarcoma viruses made by fractional centrifugation of tumour extracts and enzyme digestion (Amies & Carr, 1939; Carr & Harris, 1951) gave slightly cream-coloured suspensions and yellow-brown pellets when centrifuged, in contrast to most other viruses, e.g. vaccinia, concentrates of which are a dead chalky white. It was thought that the colour of the former might indicate the presence of an enzyme, a suspicion reinforced by the fact that these viruses are the only ones that are destroyed so rapidly by oxidation (Mueller, 1928; Gye & Purdy, 1930) that all but the most rapid experiments must be done in the presence of an oxidase inhibitor. The present work was undertaken to study this coloured material.

EXPERIMENTAL

Coloured preparations were obtained from all the fowl tumour virus preparations studied, that is: Rous I, Duran-Reynals B, D and V, Des Ligneris, Fujinami and MH2. The details of the methods of concentrating varied somewhat (see Amies & Carr, 1939; Carr & Harris, 1951). The 'normal particles' or macromolecular preparations which result by applying similar methods to normal fowl tissues or to two chemically induced and non-filterable fowl sarcomas (obtained by courtesy of J. McIntosh & F. R. Selbie, and P. R. Peacock) appeared to be colourless. The colour of different batches of virus varied, and records for the two most closely studied, Rous I and Duran-Reynals D, showed that there was a strong correlation of depth of colour with virus activity, those tumours which yielded most virus per gram of tissue gave preparations of the deepest colour, while inactive or almost inactive preparations were almost white. This, together with the finding for non-virus particles, seemed to confirm the idea that the coloured material was an integral part of the infective virus. Chemical and biochemical studies undertaken on the basis of this idea proved abortive and need not be described. The approach next described was more informative.

In the subsequent work, the following standard procedures were used.

Virus preparations were always made by treating an aqueous 10% (w/v) extract of macerated tumour with a hyaluronidase preparation to decrease the high viscosity, clarifying on a Servall angle centrifuge at 4000 r.p.m. for 15 min., and centrifuging the supernatant for 40 min. at 12,000 r.p.m. The pellet was resuspended and clarified at 1000 g for 10 min. on a horizontal centrifuge. If then treated with crystalline trypsin and redeposited at high speed, the resuspended pellet is referred to as 'purified' virus.

The benzidine test was made with a saturated solution of freshly recrystallized benzidine in acetic acid followed by the addition of '10 volume' hydrogen peroxide solution.

Buffers used were veronal (0.1M-veronal + 0.1M-HCl) and glycine (0.1M-glycine in 0.1M-NaCl + 0.1M-NaOH).

Infectivity titrations were made by inoculating 0.2 ml. volumes of decimal dilutions of virus suspension into young chicks (Carr & Harris, 1951).

It will be convenient in the subsequent sections to use the term haemin for the chemically pure compound when used, and haematin for natural material which gave a benzidine test but was not further characterized.

Presence of haematin on particles

Most of the virus-induced fowl tumours are very rapidly growing and malignant in susceptible hosts; the tumour tissue contains many haemorrhagic spots and often large clots of blood in various stages of destruction. The latter are formed presumably when the vascular system fails to extend as rapidly as the tumour tissue in which it is embedded and is thereby ruptured. The presence of old blood implies the presence of haematin which is known to adsorb very strongly to almost any protein. It was therefore suspected that a part of the colour of the virus preparations might be due to such adventitious haematin. A preparation of 'purified' Rous I virus gave a strongly positive reaction with the benzidine test. Centrifugation experiments indicated that the material reacting with benzidine resided solely in the particles; the supernatants after deposition of the particles were inactive to the reagent. Similar tests showed that preparations of Duran-Reynolds D, Fujinami and MH2 virus also contained haematin, as did particles derived from the non-filterable GRCH/15 sarcoma of Peacock, and from normal spleen, thymus, heart, muscle, or bursa of Fabricius. Though the benzidine test is not a quantitative one, the reactions of the latter preparations were clearly very much weaker than those of the virus preparations. It was concluded that these preparations too contained haematin in small quantities, although insufficient to give an obvious brown colour to the particles. The apparent correlation of the colour of the virus preparations with their activities seems therefore to be merely a reflexion of the growth-rates of the tumours from which they were derived, for most virus is harvested from rapidly growing tumours which are also usually the most haemorrhagic (Carr, 1953). The GRCH/15 sarcoma and the normal tissues contain by comparison relatively little blood, and no old clots.

Adsorption of haemin

Attempts to remove haematin from the virus preparations and yet leave them infective were unsuccessful. Centrifugation at various pH values in veronal and glycine buffers up to the limits of tolerance of the virus (pH 4–11), and extraction of freeze-dried virus or tumour with petrol ether, ether, hexane, benzene, chloroform or dioxan, failed to remove the colour. Paper-strip chromatography with various aqueous salt solutions as the moving phase, or paper strip electrophoresis at pH values of 5, 7 or 9, all failed to separate the haematin and the protein components. More drastic methods destroyed the virus, and even then the haematin remained attached to any protein or denatured protein matter still left, and did not go into solution.

On the other hand, virus preparations readily adsorbed much more haemin. A preparation of Rous 1 virus from 40 g. tumour was resuspended in 20 ml. Lemco broth + 20 ml. water, and two samples of 12.5 ml. were taken. To one sample an equal volume of 0.1 M-veronal was added, and to the other an equal volume of 0.1 M-veronal saturated with haemin hydrochloride. The preparations were deposited at 12,000 g in the angle centrifuge; the haemin-treated material gave a pellet which was black. The pellets were resuspended in 12.5 ml. Lemco broth (i.e. 1 ml. = 1 g. tumour), and on titrating in young chicks they were found to be equally potent and equal in potency to the original preparation; they gave tumours at a dilution corresponding to 0.2×10^{-4} g. of tumour. This type of experiment was done seven times with variations in the 'purity' of the virus (i.e. inserting a stage of tryptic digestion) and in the number of washings the pellets received after staining with haemin. Always the infectivity was unaltered by the adsorption of haemin, and the pellets always remained black.

Haemin adsorbed on Rous 1 virus in this way was as firmly attached as the natural colour, and was not to be removed by any of the methods that had been tried for the removal of the haematin. It did, however, appear slightly to modify the physical behaviour of the virus preparations. The haemin-stained material seemed to yield a more sticky and coherent pellet after centrifugation, and resuspension to give dispersed particles was noticeably more difficult. Since this phenomenon might have affected some of the methods used to 'purify' various virus preparations, it was studied in more detail.

Effect of haemin on physical properties

A preparation of 'purified' Rous 1 virus was made and divided into two parts, one of which was stained with haemin in the manner previously described. Each portion was separately clarified at 1000 g on the horizontal centrifuge, and the two portions then united. This material was submitted to three cycles of alternate fast and slow centrifugation, after the manner usually recommended for the purification of viruses. It was noted that at each deposition the stained material tended to collect at the bottom of the pellet, probably because of the extra specific gravity conferred by the iron of the haemin molecules. Furthermore, since this material resuspended less

readily, it was possible to stop resuspension at a stage when the unstained material was more dispersed than the stained, and on clarification at 1000 g the deposit contained more of the haemin. Thus by partial separation at each stage, three cycles were enough to separate the two fractions almost completely. Such coloured material is often discarded as 'impurity' by such cyclic processing in the purification of viruses by fractional centrifugation. In the present instance a loss of 50 % of active virus resulted from the attempt to remove a fraction of a percentage of harmless haemin, and that incompletely. This suggests that such 'purification' is wasteful and unnecessary in many cases.

Effect of haemin on oxidative destruction of virus activity

Though haemin had thus been shown to have no immediate effect upon the virus, it remained possible that its presence might affect the stability of the virus to oxidation. There does not seem to be any evidence that the destruction by oxidation is due to an enzyme contained in or adsorbed by the virus; oxidative destruction has been shown only in tumour extracts, and it is possible that any enzymic reaction is due to the tissue extract itself. In any case adsorbed haemin (which may act like catalase) might modify the result, either protectively by destroying peroxide or destructively by liberating 'active' oxygen at the surface of the virus particle. The following experiments were therefore carried out.

In each experiment, a preparation of 'purified' Rous 1 virus was made and divided into two parts, one of which was stained with haemin as described above, washed by depositing the virus once more from the suspension, and resuspended in the medium required. The unstained portion was treated in the same way except that haemin was not added. The two parts were titrated and always found to have equal amounts of active virus before further treatment.

Destruction at 37°. The stained and unstained preparations were resuspended in Lemco broth to concentrations such that 1 ml. \equiv 2 g. tumour. Two samples of 5 ml. were placed in 2 oz. McCartney bottles; to one set was added 5 ml. of Lemco broth and to the other 5 ml. of the original tissue extract from which the virus had been deposited, thus reconstituting the clarified tumour extract diluted with Lemco broth. All bottles were loosely stoppered and incubated for 20 hr. at 37°. At the end of that time they were tested and found to be bacteriologically sterile, and the activity had fallen from 10^4 tumour-inducing particles/ml. to zero in all four bottles. Inactivation of 'purified' virus therefore occurred independently of the presence of the tissue extract, and the presence of haemin did not affect this in either case.

Protection by HCN at 37°. The stained and unstained preparations were resuspended in the extract from which the virus had been deposited, and three samples of 5 ml. of each placed in McCartney bottles. To each was added respectively 1 ml. of HCN at concentrations to give final amounts of 1/10,000, 1/20,000 and 1/40,000, and then to each bottle a solution of 400 I.U. penicillin in 0.2 ml. After incubation at 37° for 20 hr. with loose stoppers, no loss of virus activity was found in any bottle, and all were bacteriologically sterile.

Destruction at 0° for 5½ and 30 hr., and at 37° for 5½ hr. The stained and unstained preparations were resuspended in Lemco broth at a concentration such that 1 ml. \equiv 2 g. tumour and two 5 ml. samples of each placed in loosely stoppered McCartney bottles. One set was incubated at 37°, the other set placed in a refrigerator at 0°. Each was titrated after 5½ hr. when an activity of c. 10 % of the original was found in the specimens incubated at 37°, while those incubated at 0° were substantially unaltered. The latter were tested again after a total of 30 hr. at 0°, when only a slight fall in activity was shown by both the stained and unstained specimens.

Destruction at room temperature (c. 14°). Stained and unstained specimens were resuspended in Lemco broth containing 400 I.U. penicillin at a concentration of 1 ml. \equiv 2 g. tumour, and left in McCartney bottles on the bench. After 20 hr. a 90 % fall in activity was found to have occurred in each bottle, and a further fall to about 1 % of the original activity value was found after a further 20 hr. for each specimen. Under all conditions tested, therefore, the stained and unstained preparations were equally sensitive to oxidative destruction, and equally protected by HCN.

DISCUSSION

Since it has been shown that the fowl virus preparations and macromolecular preparations from normal and malignant tissues give a benzidine reaction like haematin, and that haemin will strongly adsorb upon such virus preparations, it is reasonable to conclude that much of the haematin on the particles is derived from the host blood. The correlation of depth of colour of the particles with the amount of blood in the tissue supports this. The absence of other pigment in the particles has not been proved. Even if blood-derived haematin could be eliminated (e.g. by using tissue culture material), the tumour cells themselves would contain haem pigments, e.g. cytochrome, so that preparation of virus free from haematin is perhaps impossible, even if the pigment is purely adventitious.

Since haematin has a catalase-like activity, the reports that catalase is associated with these fowl-tumour viruses require reconsideration. Catalase activity has been reported for Rous 1 by Stern & Duran-Reynals (1939), and for erythroleukaemia virus by Stern & Kirschbaum (1939), though these workers were uncertain whether the activity was due to the viruses themselves or to an 'associated substance'. The present work suggests that much, perhaps all, of the catalase activity in their virus preparations could have been due to adsorbed haematin, and that no catalase proper was involved. Catalase activity was reported for vaccinia preparations by Macfarlane & Salaman (1940); Hoagland, Ward, Smadel & Rivers (1942) pointed out that catalase was strongly adsorbed by vaccinia from a dilute solution of the enzyme. Neither group of workers seems to have considered the possibility of haematin as a possible contaminant of their vaccinia virus preparations. It is unlikely that a decision as to whether the catalase activity is due to an enzyme or to haemin can be reached by determining the catalase activity of virus preparations. Though the activity of haemin is much weaker than most catalase

enzymes on a molecular basis (Kreke, Bartlett & Smalt, 1945), this may not apply when the haemin is associated with a protein, for at least one haem-protein complex is catalase itself. Even if this complication be ignored, there is room for relatively many more haem molecules than catalase molecules on the surface of a virus particle. In addition, it is unlikely that a catalase molecule could penetrate inside a virus particle, while the depth of colour of the haemin-stained virus preparations indicates that the haemin molecule is probably contained inside as well as outside the particle. The much greater quantity of haemin that a virus particle can carry might compensate for its decreased activity. The presence of haematin implies the presence of iron, yet investigations on vaccinia virus preparations (Hoagland *et al.* 1940) and normal macromolecular protein (Claude, 1941) indicate the presence of copper. It is, of course, possible that iron-containing particles were discarded during purification by fractional centrifugalization, as was shown to occur in the present work. Furthermore, porphyrin compounds are notorious for picking up copper during processing of any kind. Some of the evidence for the absence of iron in the vaccinia preparations, e.g. no inhibition of cysteine oxidation in the presence of vaccinia virus preparations when $\alpha\alpha'$ -dipyridyl was added, is evidence only for the absence of ferric iron; haem iron is in the ferrous state. The diethyldithiocarbamate test for copper could not be applied to the intact Rous virus preparations as used in the present work, for the intense brown of haem iron is as strong as the expected brown from an equivalent amount of copper with this reagent.

There may be no discrepancy in the present finding of haem iron in fowl-tumour virus preparations and the finding of copper in other materials which, as mentioned earlier, usually give whiter preparations. The finding of haematin implies that caution should be exercised in claiming the presence of catalase in viruses without first testing for haematin; and that in testing for iron, ferrous as well as ferric iron should be looked for.

The separation of the haemin-contaminated and ordinary Rous virus by the common method of virus purification, namely, alternating cycles of slow and fast centrifugation also suggests that the value of this process might well be reconsidered. In the experiment done in the present work, only a fraction of the original very small amount of harmless haematin was discarded by the sacrifice of half of the actual virus prepared by rather tedious and lengthy methods.

All expenses in connexion with this work were borne by the British Empire Cancer Campaign.

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(Received 10 November 1952)

Requirements for the Growth and Sporulation of *Trichophyton persicolor*

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SUMMARY: The optimum temperature for the growth and sporulation of *Trichophyton persicolor* was 25-30°; 40° inhibited growth. A range of pH values from 4 to 10 was compatible with growth, the optimum pH value being slightly less than pH 7. Macroconidia were produced over a narrower range and were most abundant at pH 8. Percentage germination and length of germ tube were greatest at 100 % humidity. No germination occurred at humidities of below about 95 %. Exposure to light had no effect on growth rate, sporulation or pigmentation.

The carbon compounds which supported best growth were mannose, glucose, mannitol and maltose. Nitrates were not utilized, and ammonium sulphate, urea and asparagin supported poor growth. The amino-acids leucine, glycine, histidine and cysteine were utilized; with all other single amino-acids tested, growth was negligible. Methionine and phenylalanine were inhibitory.

T. persicolor was autotrophic for vitamins. It grew as well on a vitamin-free medium as on the basal medium.

The elements zinc, iron, copper and manganese, as sulphates, stimulated the growth and sporulation of *T. persicolor* on a medium treated for the removal of trace elements.

This investigation arose from an observation that two isolates of *Trichophyton persicolor* (M2, isolated in France, and M3, isolated in Britain) grew very differently from each other on Sabouraud's 'conservation' medium in which a peptic digest of meat replaced commercial peptone, while on Sabouraud's maltose 'proof' medium containing the same digest as nitrogen source their cultural appearance was similar. As no detailed investigation of the physiology of *T. persicolor* had been made previously it was decided to make a study of the nutritional and physico-chemical growth requirements of M2 and M3. Other isolates of *T. persicolor* were frequently included for comparison. Among these were one (M 323) from Dr R. Vanbreuseghem (Institut de Médecine Tropicale Prince-Léopold, Anvers), four (M 330, M332, M333 and M335) from Dr Jacqueline Walker (London School of Hygiene and Tropical Medicine) and two (S45 and S78) recently isolated from material sent to this laboratory.

No explanation of the difference between M2 and M3 has emerged during the course of this work, but an outstanding effect of environmental conditions of the production of macroconidia has been frequently noted.

T. persicolor Sabouraud (*Les Teignes*, 1910, p. 371) has been isolated only from human ringworm infections of the glabrous skin and there are few records outside western Europe. It has never been found to infect the hair or hair follicles of man or of experimentally inoculated guinea-pigs.

On sugar media such as beerwort agar or Sabouraud's glucose agar, cultures of the experimental isolates were granular, and light buff coloured, brownish

in reverse. On such media they rapidly became pleomorphic, a white cotton-wool-like mycelium overgrowing the colony. On Sabouraud's conservation medium (containing 30 % w/v peptone but no sugar) the colonies were velvety and rose-violet, and in reverse deep wine colour. Microscopically, numerous microconidia ('en grappe' and 'en thyse'), spirals, and chlamydospores were observed. Macroconidia, which were most abundant in older cultures, were large (23×4 to 42×7 (av. 35×5) μ .), smooth and thin-walled. This appearance agrees well with that described by earlier workers (see Vanbreuseghem, 1949) except that no nodular organs were ever seen.

Apart from the production of a peach-coloured pigmentation on media of low sugar content, *T. persicolor* clearly closely resembles *T. mentagrophytes*, of which some workers consider it to be only a variant. However, *T. persicolor* has been regularly recorded in Europe since it was first described and does appear to merit a special designation. Here, the original name is adopted, but for those who prefer it, the combination *Trichophyton mentagrophytes* var. *persicolor* has been proposed by Magalhães (1935).

METHODS

The basal medium used for routine experiments was: K_2HPO_4 , 1.0 g.; KCl, 0.5 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; $FeSO_4 \cdot 7H_2O$, 0.01 g.; glucose, 20.0 g.; peptone, 2.2 g.; distilled water to 1000.0 ml. This is a modification of the Czapek-Dox solution, with peptone replacing the sodium nitrate at an equivalent nitrogen level and with glucose as the carbon source. The medium was sterilized by autoclaving at 10 lb./sq.in. for 15 min. The final pH value was 6.4-6.6, which is approximately optimal, and therefore no adjustment was made.

For preliminary work solid medium was used, the basal medium being solidified with 2 % agar. For more detailed quantitative studies the fungi were grown on 25 ml. volumes of liquid medium in 100 ml. Pyrex conical flasks. These cultures were incubated for 2 weeks at 25° and the mycelium then filtered off on to dried, weighed filter-papers, washed with distilled water, and dried to constant weight at 100°. Each treatment was carried out in triplicate and each experiment repeated at least once. The macroscopic and microscopic appearance of the colonies and the final pH values were noted at the end of each experiment.

RESULTS

Physico-chemical factors

Temperature. The effects of temperature on growth were examined by incubating cultures on liquid and solid media at room temperature and at 20, 25, 30, 35 and 40°. Readings (dry weights or colony diameters) were taken at intervals of three days. The optimum temperature for the growth of both M2 and M3 during 4 weeks was 25-30° and the development of micro- and macroconidia was most abundant in this range. At 35° growth was very wrinkled, and numerous chlamydospores, but few or no conidia, were produced. Incubation at 40° was inhibitory and prolonged exposure to this temperature was lethal; cultures transferred to 25° after 2 weeks at 40° did not

grow. In this respect *T. persicolor* differed from a strain of *T. mentagrophytes* which grew best at 35° and showed fairly good growth at 40° (Fig. 1). Robbins & Ma (1945) also found 35° to be optimal for *T. mentagrophytes*.

Hydrogen-ion concentration. The pH value of the basal medium was adjusted most satisfactorily by the addition of sulphuric or tartaric acids for the lower pH values and of sodium carbonate for pH 7–10. These substances are not utilized as nutrients by *T. persicolor* and are not toxic at the concentrations required. They held the pH value of the medium at a constant level for the duration of the experiment. When sodium hydroxide was used in place

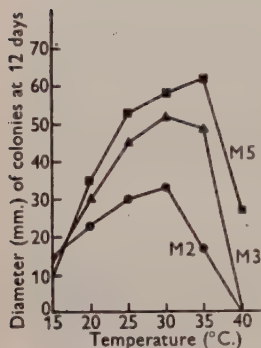


Fig. 1. Effect of temperature on growth of *Trichophyton persicolor* (M2, M3) and *T. mentagrophytes* (M5)

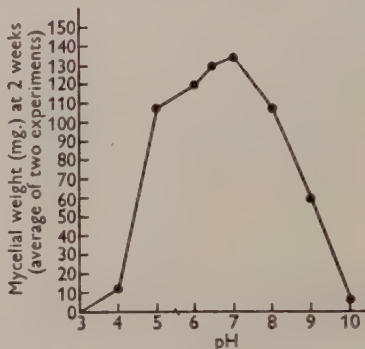


Fig. 2. Effect of pH value of medium on the growth of *Trichophyton persicolor* (M2).

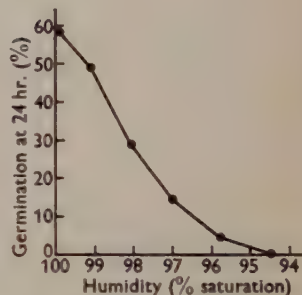


Fig. 3. Effect of humidity on spore germination of *Trichophyton persicolor* (M2).

of sodium carbonate the pH value dropped rapidly because of the absorption of carbon dioxide from the atmosphere. This gave an abnormally high result for the growth in the alkaline range. Britton & Wreford's citric acid and potassium dihydrogen phosphate buffers (Britton, 1932) were also found to be unsuitable. The pH value of the medium was adjusted as required by the use of a Cambridge pH meter or of a Lovibond comparator. The pH value of the medium at the beginning and end of each experiment was determined by means of the British Drug Houses Ltd. capillators.

Growth was studied over a range of pH values from 3 to 10, at intervals of 1 pH unit. *T. persicolor* resembled other dermatophytes in growing in a pH range of 4–10, the optimum being neutral to slightly acid (Fig. 2). At pH 4 and at pH 10 growth was very poor and few or no microconidia were produced. Microconidium formation was greatest at the optimum pH value for growth. Macroconidia were produced over a smaller range, and were most abundant at pH 8. There was a striking increase in the formation of macroconidia by M2 and M3 with increasing pH value. This effect was also noted, though to a lesser extent, for other strains of *T. persicolor*, but not for isolates of *T. mentagrophytes* and *Microsporum canis*.

The reverse pigmentation of M2 and M3 in liquid culture changed from orange brown in acid to bright yellow in alkaline media.

Humidity. The effect of humidity on spore germination was determined by using the technique devised by Davidson & Gregory (1934). Spores of several strains of *T. persicolor* were placed on dry sterile cover-slips and inverted over van Tieghem cells in which the humidity was controlled by use of sucrose solutions of varying concentrations. The concentrations of sucrose and the resultant humidities were:

Concentration of sucrose solution (moles/l.)	0.0	0.5	1.0	1.5	2.0	2.5	3.0
Calculated humidity of atmosphere (% saturation)	99.92	99.08	98.09	96.99	95.81	94.60	93.30

The cells were incubated at 25° and examined for germination after 24 hr. Results are shown in Fig. 8. Germination and length of germ-tube were greatest at 100 % humidity (over pure water) and decreased rapidly with decreasing humidity. No germination occurred at humidities below about 95 %.

Light. Strains of *T. persicolor* on Sabouraud's conservation medium, beer-wort agar or basal agar were grown in complete darkness or in daylight. Examination after 1 month showed that exposure to light had no apparent effect on growth rate, pigmentation or spore production.

Nutritional requirements

Carbon. *T. persicolor* grew on the basal medium without any other carbon source than that provided by the peptone, but at the low concentrations of peptone used growth was greatly stimulated by the addition of certain carbon compounds. The following compounds were tested for their ability to support growth by using them in place of glucose in the basal medium at 20 g./l.; the numbers in brackets following the names of the compounds are the dry weights in mg. of the mycelium of strain M2 obtained in one representative experiment on liquid medium in which the mycelial dry weight without added carbon was 8 mg. *Pentoses*: xylose (12), rhamnose (6); *hexoses*: glucose (130), mannose (146), galactose (18); *disaccharides*: sucrose (10), maltose (70), lactose (13); *trisaccharide*: raffinose (13); *polysaccharides*: starch, glycogen, cellulose (ashless filter paper); *sodium salts of organic acids*: acetate (8), citrate (7), tartrate; *alcohols*: glycerol (28), mannitol (92); *glucoside*: salicin (37).

The compounds which supported best growth, in order of availability, were thus: mannose > glucose > mannitol > maltose. The weight of mycelium produced on maltose, the only disaccharide utilized, was only half that produced on glucose. Glycerol and salicin caused slight stimulation of growth of both M2 and M3, while galactose was utilized slightly by M3 only. All other compounds tested did not encourage greater growth than on the basal medium alone.

On media containing mannose, glucose or mannitol *T. persicolor* was buff-coloured and the pH value of the medium remained constant for the duration of the experiment. On all other media, including maltose, it was peach-coloured and the pH value rose to 8, as on the medium without added carbon.

On the salicin medium M2 always produced large numbers of macroconidia. This effect was not observed when salicin was added at concentrations of 1 or 10 g./l. to Sabouraud's conservation or proof media, or to beerwort agar. Salicin did not stimulate the formation of macroconidia by other isolates of *T. persicolor* tested.

Thus *T. persicolor* does not differ markedly in its carbon nutrition from other dermatophytes. Mannose, glucose, mannitol and maltose have been found by other workers to support the growth of all species of dermatophytes tested; glycerol, salicin and galactose are not utilized so widely. A slight but definite utilization of sucrose was reported for *T. interdigitale* (Goddard, 1934; Mosher, Saunders, Kingery & Williams, 1936) and for *Microsporum canis* (Goddard, 1934; Giblett & Henry, 1950), but this was not observed in the present experiments.

The effect of varying the glucose:peptone ratio was studied over a range of concentration of 0–40 g. glucose and 0–30 g. peptone per litre. With increasing glucose concentration colonies became more spreading and pleomorphic. With increasing peptone they became more compact with a clearly defined edge and deep radial furrows. The formation of the peach-coloured pigment was controlled by the carbon:nitrogen ratio, increasing with increasing peptone and decreasing with increasing glucose. For example, at 40 g. glucose/l. and 10 g. peptone/l. (as in Sabouraud's proof medium) colonies of *T. persicolor* were cream-buff in colour while at 40 g. glucose/l. and 20–30 g. peptone/l., they were deeply peach-coloured. Microconidia were produced in large numbers at all concentrations, but macroconidia were formed most abundantly at relatively high concentrations of peptone.

Nitrogen. The compounds sodium nitrate, ammonium sulphate, urea, and asparagine were tested for their ability to support the growth of M2 and M3 by using them in place of peptone in the basal medium at an equivalent level of nitrogen (i.e. equivalent to 330 mg. N/l.). Nitrate was not utilized and growth on media containing the other three compounds was very poor. Pleomorphic forms of *T. mentagrophytes* have been found to grow more readily than normal forms on ammonium media (Robbins & Ma, 1945; Robbins & McVeigh, 1946; McVeigh & Campbell, 1950). Ammonium sulphate was almost completely unavailable for all of the 12 isolates of *T. persicolor* tested, including several pleomorphic forms. It was concluded, therefore, that nitrogen in the form of amino-acids was necessary for growth.

Growth was studied on the following amino-acids: β -alanine, glycine, L-histidine HCl, DL-valine, L-leucine, DL-methionine, DL- β -phenylalanine, L-cysteine, L-tyrosine, and monosodium glutamate. These were added to the basal medium singly in concentrations of 0.2 and 2.0 g./l. When mixtures of the acids were used, the amounts of individual acids were such that the final total concentration of acids was 2.0 g./l., divided equally among the acids in the mixture. Cystine and tyrosine are insoluble and were therefore added as suspensions.

Growth on each of the amino-acids at both concentrations was very poor. Leucine and glycine were the only acids which supported fair growth and

sporulation, and there was an appreciable decrease when either was omitted from an otherwise complete mixture. Histidine and cysteine were utilized best at the lower concentrations. With all the other amino-acids growth was very poor and almost entirely subsurface in liquid media; no growth occurred with methionine or phenylalanine. A complete mixture was not very beneficial and there was an increase in growth when methionine and phenylalanine were omitted. These acids were thus apparently inhibitory at the concentrations used. A mixture of leucine, glycine, histidine and cysteine at a total concentration of 2.0 g./l. was most beneficial (mycelial dry weight, 45.0 mg.), though growth on this did not approach that on peptone media (153.0 mg.).

These results agree fairly well with those of other workers. Leucine was found to be nearly indispensable for *T. interdigitale* (Mosher *et al.* 1936), and leucine and glycine were included by Robbins & Ma (1945) among the acids which gave best growth of *T. mentagrophytes*. These authors also found that growth on methionine was very poor and a mixture of five amino-acids, including this one, was inhibitory. All of the dermatophytes studied by Archibald & Reiss (1950), including *T. mentagrophytes*, grew very poorly on methionine alone. Phenylalanine was found by McVeigh & Campbell (1950) to be toxic to *T. mentagrophytes* at 10.0 g./l. (i.e. at an equivalent level of nitrogen to that supplied by 2 g./l. asparagine).

Considerable variation was found between different brands of peptone. In one experiment the dry weight of mycelium produced by M2 on four different peptones was 43, 72, 86 and 124 mg., respectively. Variation between different batches of the same brand was as great as that between different brands. The peptone used at the beginning of this investigation supported good growth of *T. persicolor*, but a later supply was completely unsatisfactory. When used in Sabouraud's conservation medium it gave rise to poor unpigmented colonies which were deeply folded and produced only chlamydospores. This emphasizes the need for a more complete knowledge of the nitrogen nutrition of dermatophytes.

Growth factors. *T. persicolor* grew as well on a basal medium containing vitamin-free casein hydrolysate as the nitrogen source as on a peptone medium. Yeast extract, added to vitamin-free casein hydrolysate, ammonium sulphate or asparagine media at concentrations of 0.01, 0.1, 0.2 and 0.3 % (w/v) stimulated growth. This stimulation was no greater than that caused by the addition of a quantity of vitamin-free casein hydrolysate containing an equivalent amount of nitrogen (micro-Kjeldahl). Addition to vitamin-free media of thiamine, nicotinic acid and riboflavin at a concentration of 500 µg./l., singly or in combination, did not stimulate growth. *T. persicolor* is thus apparently autotrophic for vitamins but requires organic nitrogen for growth. Isolates of *T. mentagrophytes* studied by Burkeholder & Moyer (1943) and by Robbins & Ma (1945) were also auxo-autotrophic, while the strain investigated by Arêa-Leão & Cury (1950) required added thiamine and inositol.

Trace elements. Steinberg's method of removing trace elements was used (Steinberg, 1919). The basal medium was autoclaved at 15 lb./sq.in for 15 min. with 15 g. calcium carbonate/l. and was filtered hot through ashless filter-paper.

All glassware was cleaned before use with chromic + sulphuric cleaning mixture and washed with glass-distilled water. The elements zinc, iron, copper and manganese, as sulphates, were added singly or in combination at concentrations of 0.2, 1.0, 5.0 and 20.0 parts of the element per million. In addition, the concentrations of these elements recommended by Steinberg for *Aspergillus niger* (i.e. Zn, 0.18; Fe, 0.20; Cu, 0.04; and Mn, 0.02 p.p.m.) were used (from Foster, 1949). Batches of medium as made up were tested for removal of trace elements by growing on them a strain of *A. niger* which showed very poor colourless sporulation on a similarly treated medium containing ammonium nitrate in place of peptone.

Table 1. *Trace element nutrition of Trichophyton persicolor*

Medium	Experiment					
	1	2	3	4	5	6
	Average weight of mycelium (mg.)					
Treated	21	171	143	31	86	94
Treated + Mn, Zn, Fe, Cu	101	150	126	123	2	121
Treated + Mn	71	193	141	180	95	113
Treated + Zn	68	184	174	173	91	127
Treated + Fe	70	172	171	56	68	113
Treated + Cu	55	130	120	83	83	104

(Elements added at 0.2 p.p.m.)

The results obtained were very inconsistent. In some experiments they were significant, growth being negligible in treated solutions without added trace elements and greatly stimulated by their addition. In other experiments the difference in weight on treated and untreated solutions was not very marked (Table 1). In all experiments the effect of the partial removal of trace elements on the appearance of growth and spore production of *T. persicolor* was very noticeable. Even when the growth rate was good on treated solutions the mycelium was almost entirely subsurface and no spores were produced. On treated media with added trace elements growth and sporulation were as good as on the untreated solution. The lowest concentrations of trace elements used were sufficient to meet the requirements of *T. persicolor*; there was no further increase of weight or of sporulation with further increased concentrations.

It was thought that inconsistencies in results might be due in part to the trace element content of the inoculum. Suspensions in glass-distilled water of fungi which had been cultivated on media 'free' from trace elements were therefore used for inoculation, and the effect of using very small amounts of inoculum was also determined. Results were no more constant when either of these precautions were taken. The variations were therefore presumably due to the difficulty in removing traces of elements from the peptone (see Donald, Passey & Swaby, 1952). Solutions were also treated for removal of trace elements by passing them through a column of alumina. This had some effect, growth being greater on a treated medium to which elements had been

added than on one without added elements, but the method was not so effective as Steinberg's.

Isolates of *T. mentagrophytes* (Robbins & Ma, 1945; Robbins & McVeigh, 1946; McVeigh & Campbell, 1950) have been reported to grow well with nitrogen supplied as ammonium salts. Several isolates of this species and of *T. persicolor* were tested to see whether they grew well enough on ammonium sulphate media to enable their trace element nutrition to be studied on an inorganic medium. Growth of all isolates on such media was however negligible.

The only previous study of the trace element nutrition of dermatophytes I have found is that of Mosher *et al.* (1936), who claimed to have failed to obtain growth of *T. interdigitale* on a medium from which zinc, iron, copper and manganese were omitted. Details of their methods were not given, however. This aspect of the nutrition of dermatophytes requires further investigation.

This investigation was undertaken during the tenure of a Medical Research Scholarship awarded by the Court of the Grocer's Company, to whom I wish to express my thanks.

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(Received 24 November 1952)

The Cytology and Life-cycle of *Azotobacter chroococcum*

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SUMMARY: *Azotobacter chroococcum* exists in two cytologically distinct vegetative forms. In one the appearance is that of a typical bacterial resting cell, with a large vesicular nucleus. In the other the cytoplasm appears homogeneously vacuolated and refractive; this is correlated with a high lipid content. Gonidia of two types are produced from the nucleated cells. The more common are tiny bacilli with polar flagella; these are released from the lumen of the mother cell and reproduce by fission. Larger gonidia, resembling miniature vegetative cells, develop within the mother cell, which becomes greatly elongated. These large gonidia do not appear to divide but grow directly into vegetative cells. Mother cells of both kinds may be Gram-positive.

The fact that species of *Azotobacter* possess complex life cycles has been recognized for a relatively long time (Jones, 1920; Löhnis, 1921). Several points remain to be clarified, however, notably the relationships of the different growth forms observable in typical vegetative cultures to the formation of gonidia, the nature of the gonidial granules and the importance of 'symplasm'. In the present study the cytochemistry of the two major growth forms was studied in some detail and the mode of formation of the gonidia observed. The problems of vegetative cell division and the formation and germination of cysts, which have been fully described by Pochon, Tchan & Wang (1948), were not approached on this occasion.

METHODS

Six strains with the characters of *Azotobacter chroococcum* were isolated from moistened soil plates, upon which they formed brownish mucoid colonies after about a week at room temperature. For cytological purposes the strains were grown either in tuberculin flasks in fluid medium, upon the surface of which a tough pellicle was formed, or upon a similar medium solidified with 2% agar. The composition of the medium was: 1% mannitol; 0.05% K_2HPO_4 ; 0.02% $MgSO_4 \cdot 7H_2O$; 0.02% NaCl; traces of manganese sulphate and ferric chloride; in tap water.

A wide variety of methods was used to stain these organisms. Good results were always easier to obtain when the cells were first defatted with xylene for about 5 min. at room temperature. The xylene was removed with 75% ethanol. Other solvents, such as ether, acetone and ethanol, even at boiling-point, were almost without effect.

The classical HCl Giemsa technique was unsatisfactory with vegetative *A. chroococcum*, and although the nuclear structures had previously been found to stain well with the methylene blue eosin technique (Bisset, 1948) this method was avoided, as its results were entirely unpredictable. Various agents were employed instead of N-HCl in the initial 'hydrolysis' process. The most

successful were mild alkalis in 1% solution; lithium carbonate, sodium sulphate, sodium carbonate and magnesium sulphate produced comparable but not identical results. Nitric acid was also employed. After these treatments the cells were stained with Giemsa, thionin or crystal violet. The HCl Giemsa technique was found to be very successful with gonidial forms.

Lipids were demonstrated by the Ziehl-Neelsen technique and by a modification of this method in which differentiation is conducted in a saturated solution of picric acid in 75% ethanol. Lipids were also stained by Sudan IV and by a 10% solution of sulphuric acid in acetic anhydride, which gave a transient pale violet colour; a similar result was given by 5% trichloroacetic acid.

Useful results were also obtained by the Gram method and by basic fuchsin alone, as well as by tannic acid violet for cell walls. Gold-shadowed electron micrographs were made to observe the arrangement of flagella in both vegetative cells and gonidia. These results were confirmed by silver-impregnation methods of flagella staining. Phase-contrast microscopy was also employed. No special method was used to demonstrate the polysaccharide capsule, which was not studied.

The cytochemical studies of vegetative cells are illustrated exclusively by drawings because, whereas they do not embody any original observations upon the morphology of *A. chroococcum*, the synthesis of information obtained from a very large number of stained preparations is most clearly presented in this manner. Both drawings and photomicrographs of the gonidial stages are presented.

OBSERVATIONS

Cultures of *A. chroococcum* grow very slowly for several months. In very young cultures, less than a week old, many cells have a curious appearance, sometimes described as 'vacuolated cytoplasm', in which the cell contents appear as though bubbly, full of refractile bodies like glass beads. A proportion of cells at any stage always show the typical vesicular nucleus, with a larger or smaller number of peripheral chromatinic granules. After about 10 days in culture, these forms tend to predominate until in the later stages, after several weeks or even months, the entire culture is transformed into tiny bacilli from which typical *A. chroococcum* are regenerated on subculture. The formation of gonidia is greatly accelerated in fluid medium.

The cytological staining of the nucleated forms was curiously variable and is indicated by the drawings of Figs. 1-3. When treated with a mild alkali and stained with Giemsa the entire nucleus either stained uniformly or exhibited a negative staining of the eccentric granule which, typical of bacterial resting nuclei (Bisset, 1950), occupied one or both poles of the nucleus (Fig. 1). Similar preparations stained with thionin after preliminary treatment with either alkali or acid showed the same granules staining much more strongly than the remainder of the nucleus, which appeared as an empty vesicle (Figs. 2, 3). Although, as stated above, these forms appeared to have a high lipid content, as indicated by the greater affinity of defatted material for aqueous dyes, they did not stain with Sudan IV or acetic anhydride.

By contrast, the vacuolated cells (Figs. 4–7) stained well with such lipid-indicating reagents. Young cultures examined by phase-contrast microscopy showed clearly that the difference between the nucleated and vacuolated cells was not due to staining artefacts but could be seen in living material (Fig. 4*a, b*). It is tempting to attempt to correlate the appearance of these cells, which seem to be filled with globules, with the initiation of gonidium production. There appears, however, to be no connexion; the vacuolated cells are commonest in the younger stages of culture and are remarkable only for this very high lipid content. When defatted and stained with nitric acid thionin, only the outlines of the globules appeared to stain (Fig. 5). The complementary effect was seen in preparations stained by Ziehl-Neelsen, which showed the globules to be distinctly acid-fast (Fig. 6). As a control, mixed preparations of *A. chroococcum* and *Rhizobium* sp. were stained; the latter showed no acid-fast granules. The characteristic appearance of these cells was less well seen when stained by Sudan IV or acetic anhydride (Fig. 7) as the fat globules appeared to be displaced towards the periphery of the cell. This may well have been a physical effect due to solution of the lipids. The exact nature of the lipid in *A. chroo-*

Fig. 1. Nucleated cells stained by lithium carbonate and Giemsa.

Fig. 2. As Fig. 1, by magnesium sulphate and thionin.

Fig. 3. As Fig. 1, by nitric acid and thionin. Comparison of Fig. 1 with Figs. 2 and 3 shows negative and positive staining respectively of the large perinuclear granules.

Fig. 4. Phase-contrast appearances of vacuolated cells *a* and nucleated cells *b*.

Fig. 5. Vacuolated cells stained by nitric acid and thionin. The outlines of the globules are stained.

Figs. 6, 7. As Fig. 5, stained for lipids by Ziehl-Neelsen (Fig. 6), Sudan IV (Fig. 7). In the former case the acid-fast material corresponds closely to the refractive globules seen in unstained cells, but the Sudan IV demonstrates material nearer the periphery, and possibly displaced by partial solution.

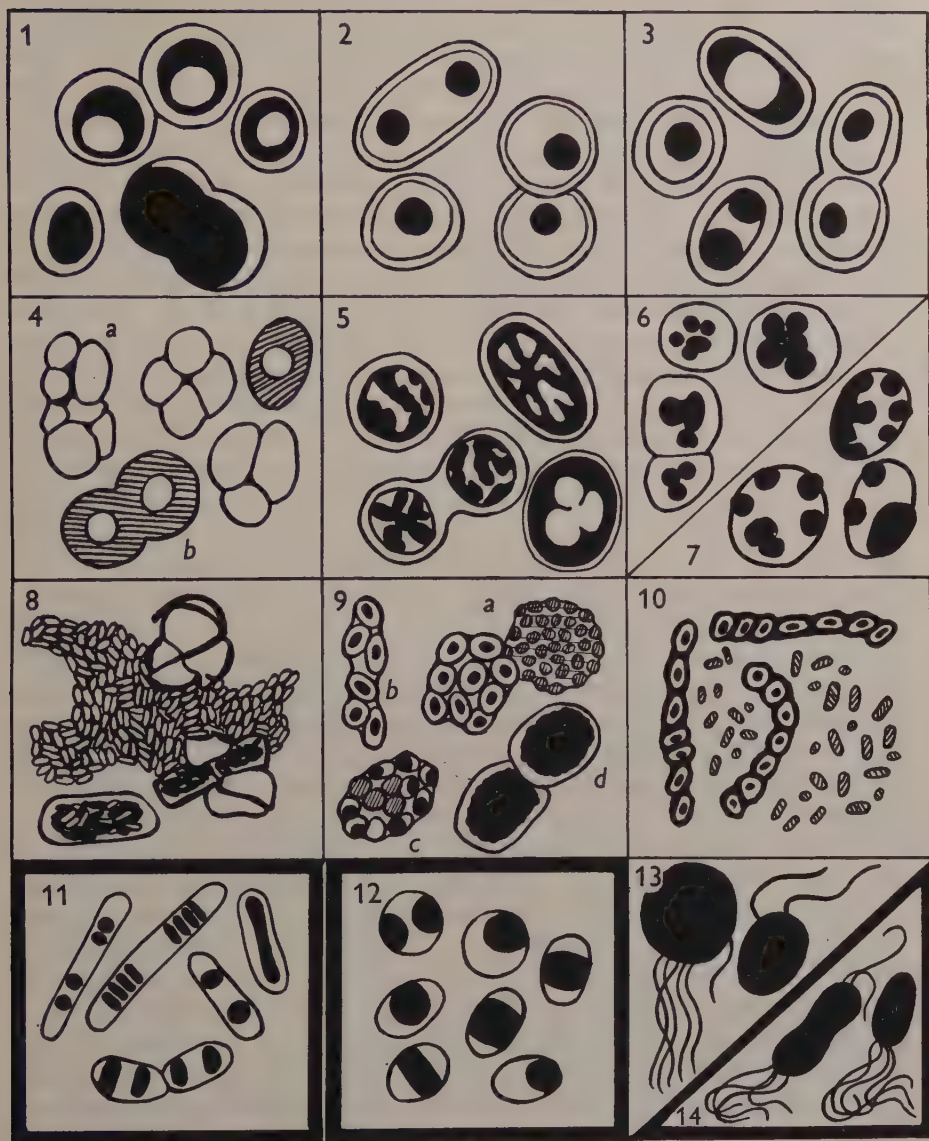
Fig. 8. Typical appearance of small gonidia released from mother cells whose broken cell walls are also shown. Stained Gram and appearing Gram-negative. Those still enclosed in the mother cell frequently appear Gram-positive.

Fig. 9. Alternative appearances seen in Gram-stained mother cells. In *a* the large type of gonidia are seen at two different stages of maturation. In *b* a relatively mature, elongated mother cell of this type is shown, by comparison with *c*, in which the postulated process of elimination of a proportion of the original, large number of granules (as in the right-hand cell of *a*) is proceeding. All these stages are Gram-negative. By contrast the mother cell *d* is filled with undifferentiable Gram-positive material, presumably immature gonidia.

Fig. 10. Free gonidia of the small type, which when released are invariably Gram-negative, as contrasted with the later stages of formation of the large gonidia, which are still contained in the now greatly elongated mother-cell, and stain with a Gram-positive cortex. With Giemsa, these chains of large gonidia stain deep red, the small gonidia, pale blue.

Figs. 11, 12. Cytological appearances of the small gonidia; rod-like and coccoid forms respectively, by HCl Giemsa. The typical bacterial nucleoids are seen.

Figs. 13, 14. Flagellation of vegetative cells and small gonidia respectively, from electron micrographs and silver impregnation preparations. The mature cells have irregularly peritrichous flagellation; the gonidia, a tuft of up to four or five polar flagella. The arrangement of the flagella at the poles of the double cell is an indication of normal cell division.



For legends see facing page

Figs. 1-14. These are drawings from microscopic preparations, with the exception of Fig. 4, which is taken from a phase-contrast study of living material, and Figs. 13 and 14 which are stylized and are from observations on both microscopic preparations and electron micrographs. All are shown at a magnification of $\times 4000$, approximately, except Figs. 11, 12 and 14 (bordered by a heavy line), which are at $\times 15,000$, approximately. The capsules are not demonstrated.

coccum is uncertain; Anderson (1948, p. 377) states that this species contains ergosterol, but since they appear to be acid-fast, and are more soluble in xylene than in ethanol, it is probable that the sterols indicated by the acetic anhydride reaction are constituents of wax-like compounds.

No sign of internal cell walls could be discerned in tannic-acid violet preparations, and it is considered that these do not occur, and that the 'vacuoles' are cytoplasmic.

Reproduction by gonidia was seen after a variable time, usually not less than 5 or 6 weeks, and cultures on solid medium did not always show the phenomenon. Curiously, it could often be initiated immediately by growth on nutrient agar containing fresh horse blood, but not boiled blood or serum. The reason for this effect is not known.

The characteristic appearance of gonidium production is shown in Fig. 8 and Pl. 1, fig. 1. Masses of tiny bacilli were seen, with the broken and empty cell walls of the mother cells, and others still packed with gonidia. Like the vegetative *A. chroococcum*, the gonidia were for the most part Gram-negative, but while still enclosed in the mother cell, a large proportion were Gram-positive. In Figs. 9, 10 and Pl. 1, fig. 1, a second type of gonidium is illustrated; Fig. 9a shows a double mother cell in which one half is occupied by small granules, apparently gonidia in the course of development, whereas the other half contains relatively large bodies exactly resembling miniature vegetative cells. Cells filled with these larger gonidia were frequently elongated in form (Fig. 9b). In Fig. 9c, however, the large gonidia are shown at an earlier stage of development, and here the cell retains its oval outline. The number is rather larger, but their appearance is variable, and some gonidia stain much less deeply and lack a defined nucleus. Since the mature mother cells of this type very rarely contained more than eight or nine gonidia, it appears that a proportion of these must degenerate to permit the development of the remainder. It is probable that the small granules seen in the right-hand portion of Fig. 9a are in a still earlier stage of development into similar large gonidia, and are consequently much more numerous than in the left-hand portion. Cells containing masses of Gram-positive material, in which no structures could be discerned, were also seen (Fig. 9d), and Gram-positivity was shown by the large gonidia at a later stage of development (Fig. 10). These did not appear to be fully released from the mother cell, but the outline of each group of six or eight cells, comprising a 'brood', became more and more elongated until they formed chains, apparently cemented by a surface material which sometimes stained Gram-positive, and which was acidophilic, staining bright red with Giemsa, whereas the small gonidia stained blue and were Gram-negative. These large gonidia apparently developed directly into vegetative *Azotobacter* on subculture, and did not reproduce; nor did they possess any demonstrable flagella.

By contrast the small gonidia appeared to reproduce freely by fission, in the course of growth. Cytologically they were typical, small bacteria (Figs. 11, 12), and possessed the nuclear structures appropriate to their morphology, whether relatively long, slender rods with paired chromosomes, or shorter

almost coccal forms with eccentric chromatinic granules (cf. Bisset, 1950). They were actively motile by tufts of from one to five flagella at the poles, in contrast to the rather irregularly peritrichous arrangement of full-grown, vegetative cells (Figs. 13, 14; Pl. 1, figs. 3, 4). On subculture these tiny rods either developed directly into vegetative forms or reproduced for some time in the form of Gram-negative rods resembling normal-sized *Bacterium* species. The large gonidia were by no means always present, and were invariably much less numerous than the small type.

The existence of 'symplasms' in the life cycle of *Azotobacter* spp. (Löhnis, 1921) is not confirmed by this study. Masses of gonidia of both types were frequently seen, and the presence of the relatively large nucleated cells within a group of tiny forms, so small as perhaps to be indistinguishable by the microscopic techniques of that date, may have given the impression of a mass of undifferentiated cytoplasm, which enclosed a number of nuclei (i.e. the large gonidia), especially if the peculiar mode of formation of the latter was not fully recognized (Pl. 1, fig. 2). It is not considered, however, that the existence of symplasms has been entirely disproved, although it certainly has not been confirmed.

DISCUSSION

The nucleated forms of vegetative *A. chroococcum* and the forms showing 'vacuolated cytoplasm' are not, as has been believed, varied appearances in similar cells produced by cytological techniques, but are distinctly different in their morphology and can be distinguished by phase-contrast microscopy of living material. The vacuolated forms appear to possess an even higher content than usual of lipid material, and are common in the early stages of culture. Despite the superficial resemblance between the refractile globules which cells of this type contain, and the gonidial granules with which mother cells become packed in the later stages of culture, there is no real connexion between the two appearances. In the nucleated stage it appears that the largest of the chromatinic granules on or near the periphery of the nucleus (Bisset, 1948) is cytochemically distinct from the remainder of the nucleoplasm, but no evidence of its nature is available.

Gonidial reproduction in this species comprises two different processes which differ fundamentally only in that whereas both probably begin in an identical manner, with the production in the mother cell of a large number of small gonidial granules, these may proceed either to the formation and release of an equivalent number of tiny rod-shaped gonidia with polar flagella, or alternatively the majority may be sacrificed to the formation of a much smaller number of relatively large gonidia which resemble miniature vegetative forms. This process is analogous to seed production in certain plants, where several fertilized ovules take part in the protection of the single one which is destined to reproduce. The small and large gonidia differ also in that the former reproduce by fission while the latter seem to be transformed directly into mature vegetative cells by a simple increase in size, are not immediately released but remain in their original groups still enclosed in the mother-cell

envelopes, and become elongated to form short chains surrounded by a cortex which frequently stains Gram-positive. The masses of developing small (or undifferentiated) gonidia within the mother cells may also stain Gram-positive for a short time before their disruption. In both cases this appearance is lost when the gonidia are released.

Whereas the large gonidia resemble the nucleated vegetative cells cytologically, the small gonidia are typical of small bacteria in this respect and are provided with a tuft of polar flagella. They are reminiscent of swimmers of *Rhizobium* spp. (Bisset & Hale, 1951), but are rather less specialized, since these are released as spherical cells with a single flagellum and develop into small bacteria at a later stage of the life cycle.

The systematic position of *A. chroococcum* has often been the subject of dispute; it has been suggested that it more closely resembles the yeasts than the bacteria, but the occurrence of such typically bacterial stages in its life cycle, together with its possession of bacterial flagella makes its inclusion in the Eubacteriales as defined by Bisset (1952) almost indisputable.

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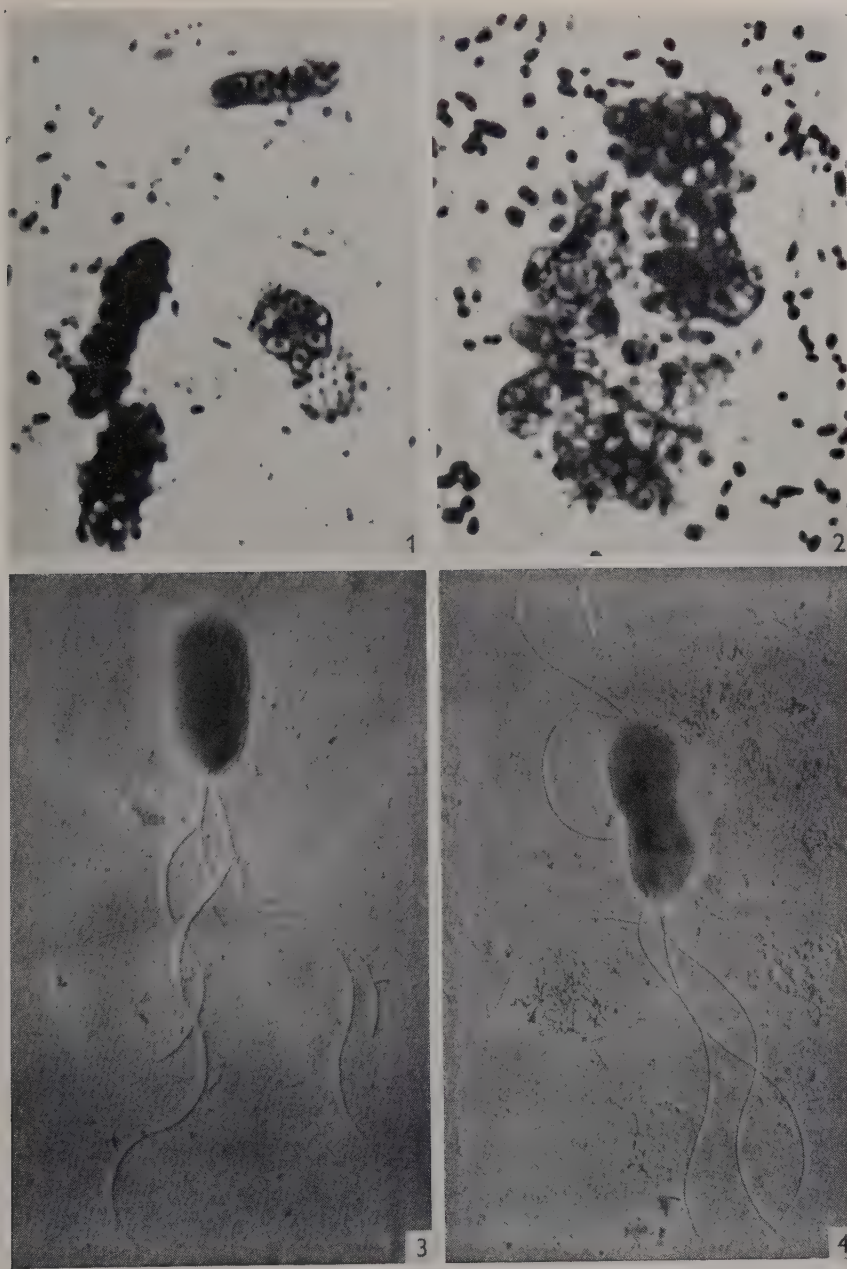
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EXPLANATION OF PLATE

PLATE 1

- Fig. 1. *Azotobacter chroococcum*; small gonidia and mother cells containing gonidia of large and small types at varying stages of maturation. Compare Figs. 8-10. Gram method; $\times 3000$.
- Fig. 2. *Azotobacter chroococcum*; 'sympiasm' showing large type of gonidia embedded in a mass of small gonidia and disrupted cell material. Gram method; $\times 3000$.
- Figs. 3 and 4. *Azotobacter chroococcum*; small gonidia showing bacillary form and polar flagellation. Electron micrograph, gold-shadowed; $\times 16,000$.

(Received 3 December 1952)



K. A. BISSET & C. M. F. HALE—CYTOLOGY AND LIFE-CYCLE OF *AZOTOBACTER CHROOCOCCUM*. PLATE 1

ERIKSON, D. (1953). *J. gen. Microbiol.* 8, 449-454.

The Reproductive Pattern of *Micromonospora vulgaris*

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SUMMARY: The thermophilic actinomycete *Micromonospora vulgaris* resembles known mesophilic members of the genus *Micromonospora* in reproducing by means of small refractile spores borne singly on lateral branches of the vegetative mycelium. Its distinctive feature is the consistent production of a secondary aerial mycelium under optimal laboratory cultural conditions and in grass composts during the high temperature phase. This aerial mycelium is composed of abundantly branched, hydrophobic filaments which rapidly segment. The cells in these filaments may in turn give rise to similar refractile spherical spores borne singly on very short lateral branches. The reproductive pattern here described is one of the most complex yet known among the actinomycetes. It is thought that the aerial phase of development is intimately associated with the thermophilic nature of the organism.

When originally defining the genus *Micromonospora*, Ørskov (1923) described the type-species, *M. chaliceae*, as completely devoid of aerial mycelium, reproduction taking place by means of small spherical to oval spores borne singly on the distal ends of short lateral branches of the vegetative mycelium. Erikson (1949) confirmed the essential validity of this description, and stated 'The few instances of aerial mycelium that have been reported... (since)... are all of the nocardial type, a sparse development of mostly unbranched and undivided filaments. Such development is spasmodic and infrequent and does not represent a sporogenous phase.' This view was in accordance with the work of Waksman, Umbreit & Cordon (1939) on the thermophilic actinomycetes which occur in dunged composts and soils. Thus, although they noted that their strains of *M. vulgaris* differed from the mesophilic strains described by Jensen (1930) by the appearance of a slight aerial mycelium on certain media, they considered this of no significance and stated that 'the aerial mycelium, though present, is usually rudimentary, rarely exhibiting the tangled network of strands typical of *Actinomyces* species'. The observation of Miehe (1907) and other early workers that the white lime-like coating on hot compost heaps was caused by thermophilic actinomycetes was re-emphasized by Forsyth & Webley (1948). Erikson (1952) explained this appearance as due to the very abundant production of an aerial mycelium which was hydrophobic and gave rise to heat-resistant spores. The brief description of the development of *M. vulgaris* which follows is based on the almost continuous observation, during a period of two to three years, of seven strains, six, M, D, F, D₃, B₂, B₄, isolated from composts, and a seventh, H, isolated from canned ham by Dr Heller.

METHODS

During experiments detailed elsewhere (Erikson, 1952) spore suspensions of suitable concentrations were continually being prepared. These were streaked on blocks of agar for slide cultures, or on strips of cellophan laid over the

water-agar medium. In other instances suspensions of spores in fresh liquid medium were run under the surface of sterile cover-slips, which were supported by discontinuous strips of agar. The practice of inverting a cover-slip over the inoculum directly on to a block of agar does not allow sufficient aeration for the development of *M. vulgaris*; similar drawbacks apply to hanging-drop cultures. The best preparations were obtained on cellophan which had been sterilized in the liquid medium and then incubated over medium of the same composition solidified by agar. An environment of 100 % humidity was provided by incubating the slide-cultures in metal slide boxes with close-fitting lids which were lined with sterile moistened absorbent cotton-wool. Incubation was at 55° or 60°.

In connexion with other work there was available a constant supply of surface and bottom growth from liquid cultures in small stoppered bottles and larger flasks; of spreading coherent growth on cellophan strips and circles; of colonies of all sizes and ages on agar plates and roll tubes. Sometimes a single giant colony, representing the survivor of a heat-testing experiment, was available. All such material was examined in the living state.

The CPS medium described earlier (Erikson, 1952; nutrient broth supplemented with casein digest, pea extract and starch) was used consistently. A simpler basal medium containing 5 % (v/v) enzymic digest of casein and Czapek mineral salts was also used, to which various additions were made, e.g. 1 % starch, maltose, sucrose or mannitol; 0.01–0.1 % yeast extract (Difco, Oxoid or Yeastrel); 5–10 % (v/v) fresh grass juice, Waring-blended grass or compost extract; 5–15 % (v/v) rabbit dung extract (made by autoclaving 1 part rabbit dung with 5 parts water); or sterilized compost.

RESULTS

Vegetative mycelium

The indispensable primary method of propagation of this organism, as of other actinomycetes, is through the proliferation of a ramified mycelium. When the submerged hyphal growing tips of an active young culture on an agar medium are removed to a suitable liquid medium, a proportion of these filaments will elongate, branch, and in 12–18 hr. fill the bottom of the container with small puffball colonies. The proportion of dismembered filaments capable of renewed life when transplanted to fresh medium is low. Very few filaments, fragmented and isolated for slide culture observation, can be noted as actually growing in any given experiment. Massive seedings are required, especially when growth on solid media is desired.

The vegetative mycelium of *M. vulgaris* differs from that of the mesophilic micromonosporas chiefly in those respects which are distinctive of thermophilic bacteria in general: (a) a greater degree of elongation of the cells, which in this type of organism leads to wider spacing between the branches and in consequence a diffuse form of growth (Pl. 1, fig. 1); (b) a more rapid disintegration of the cells, which may take place in 1–2 days rather than in the 1–2 months of the long-lived mesophiles. The rate of disintegration, however, varies

considerably according to the experimental conditions, especially as concerns degree of aeration, and the nature of the substrate and of inoculum employed. Thus, the puffball growth at the bottom of static liquid CPS medium commonly begins to segment and to decay within 1–2 days (cf. the lack of measurable oxygen uptake of this material; Erikson & Webley, 1953), and as a rule has autolysed within a week. The vegetative mycelium which spreads over the surface of cellophane sheet laid on CPS agar may still be structurally intact on the second day, even after the development of the aerial mycelium (Pl. 1, fig. 2). With a similar degree of aeration on a less rich medium, e.g. Czapek salts containing 5% (v/v) casein digest + 1% (w/v) maltose and 0.01% (w/v) yeast extract, the life of the vegetative mycelium as a whole may be prolonged for several days.

Sporulation of the vegetative mycelium

The sporulation of the vegetative mycelium differs a little from that already described for the mesophilic species. On first isolation two strains (D_3 and B_2) showed a tendency to produce spores mainly in clusters (see Pl. 1, fig. 3), especially at 55° and on compost-containing media. On further cultivation it was found that, as with the mesophilic *M. chaliceae*, 'the regular rule is single terminal spores, but any strain may show in the same field two, three, or more spores in close juxtaposition or in *Botrytis*-like clusters' (Erikson, 1941). The spores of the thermophilic strains also have the light-scattering properties characteristic of the genus. This intense degree of refractility renders the mature spores dazzling in darkground preparations, and causes them to appear as dense, opaque, spherical bodies in phase-contrast views (Pl. 1, fig. 2). Only the immature spores stain readily with the usual stains. Gray's spore stain is useful in demonstrating the older spores, which rapidly break loose from the parent branches. They are not acid fast.

Sporulation is not accompanied by a change in pigmentation as is the case of the mesophilic *M. chaliceae* whose pink-orange vegetative mycelium turns glistening brown-black as the superficial spore layer is formed. The growth of *M. vulgaris* is colourless throughout.

Aerial mycelium

Aerial filaments arise by monopodial branching of vegetative hyphae. In general the aerial filaments are slightly wider than the vegetative hyphae, as is usual with the aerial mycelium of most mesophilic streptomycetes. The aerial filaments of *M. vulgaris* rapidly branch to form an elaborately interwoven hydrophobic superstructure above the vegetative mycelium (Pl. 1, fig. 2). This aerial felt stains with Sudan Black and Sudan IV while the vegetative mycelium remains unstained. Segmentation of the aerial mycelium takes place within the first 18 hr. on most media, and can be seen very clearly by means of a phase-contrast microscope, even with a low-power objective (Pl. 1, fig. 4).

Sporulation of the aerial mycelium

Only single lateral spores are produced, at intervals, along these multicellular and often very long filaments (Pl. 1, fig. 5). These aerial spores are easily detached, usually spherical when mature, and very refractile. There seems little morphological difference between such aerially borne spores and those produced by the vegetative mycelium in static liquid cultures. It is the aerial spores which, in the tests so far carried out, have exhibited heat resistance. But it is difficult to obtain spores from vegetative mycelium alone in such quantities as are yielded by aerial mycelium.

Germination of spores

All spores germinate by one to four germ-tubes. The degree and speed of branching thereafter displayed depend upon the nature of the medium and other conditions of growth. Thus, where the new growth is in continuous contact with an adequate medium (as in liquid culture, on the surface of moist agar or on moistened cellophan overlying agar) it develops a mycelium composed of very long filaments with more or less regular branching (cf. Pl. 1, fig. 1, also Fig. 24 of Waksman *et al.* 1939). But where the supply of nutrients is locally restricted, as when a suspension of spores in broth is poured along the undersurface of a cover-slip placed at an angle over discontinuous agar blocks, it is possible to see the 'scrolls' and 'loops', which result from the elongation unaccompanied by lateral branching, of the filamentous growth, following the movements of moisture on the glass (Pl. 1, fig. 6; also compare similar patterns described by Klieneberger-Nobel, 1947, for mesophilic streptomycetes).

DISCUSSION

Compared with eubacteria, actinomycetes in general are slow of development. This is true even of the organisms which are thermophilic as in the present instance. Thus the thermophilic sporing bacilli commonly encountered in the primary isolations from the composts had completed their full development and were passing into decline before the actinomycete *M. vulgaris* had produced its aerial mycelium. This device of producing a secondary aerial mycelium, capable both of further growth and of producing spores, more than doubles the expected active life of a mycelial organism at elevated temperatures.

The observation (Erikson & Webley, 1953) that the aerial mycelium has on the whole a higher oxygen uptake at 60° than has the vegetative mycelium is another expression of the fact that *M. vulgaris* is successfully adapted to existence in the high temperature phase of vegetable composts. In competition with the more rapidly multiplying bacilli, the vegetative filaments of *M. vulgaris* would probably be at a disadvantage in obtaining sufficient space and nutrients for good growth within the films of moisture adhering to the compost fibres. Sufficient moisture is essential for vegetative growth. Examination of the compost fibres reveals a very loose straggling growth of fine

filaments with occasional portions densely sporulated. A moderately profuse mycelial development is found only where the aerial filaments branch into air spaces between the fibres.

The media described for the cultivation of *M. vulgaris* all contain complex nitrogenous substances. Two or three of the isolates when first purified were unable to produce any aerial mycelium on artificial media until these had been supplemented with rabbit dung or compost extract. It therefore seems likely that in compost heaps the first production of aerial mycelium by *M. vulgaris* has some connexion with the availability of suitable bacterial and vegetable breakdown products. The hydrophobic wall of the aerial filaments may also serve as a barrier to the passage of noxious substances, which, in the saturated humid atmosphere generally obtaining throughout the compost, might otherwise be able to penetrate the cells; or again it might protect against occasional desiccation in the superficial layers. The repetition by the aerial mycelium of the type of sporulation which characterizes the vegetative mycelium, namely, the production of single lateral spores, emphasizes this distinctive feature of the genus, which is of considerable systematic importance. The thermophilic *M. vulgaris* thus possesses properties that separate it from mesophilic species of the same genus; it appears to have one of the most complex reproductive patterns of any actinomycete yet studied.

This work was done by the author as a member of the scientific staff of the Agricultural Research Council, who also provided an expenses grant. I wish to thank Prof. J. Cruickshank for the hospitality of his department; Mr Jabez Bruce for the phase-contrast photographs; and Miss Mildred MacKay for technical assistance.

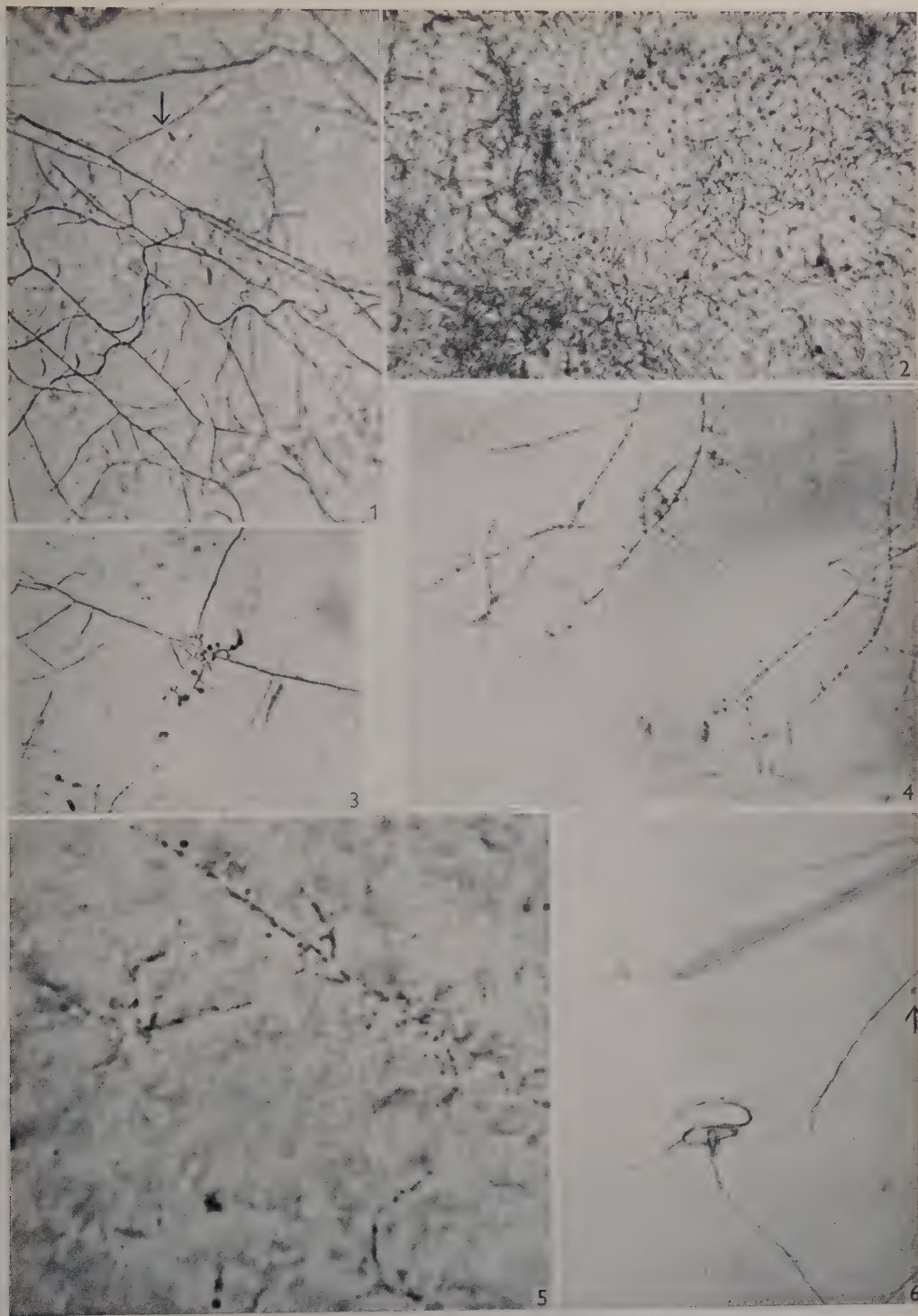
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EXPLANATION OF PLATE

- Fig. 1. Diffusely branching vegetative mycelium, 2 days old, on cellophan over CPS agar. Note single incipient lateral spore at arrow. Phase contrast. ($\times 600$.)
- Fig. 2. Another portion of the same growth as Fig. 1, showing abundant development of raised aerial mycelium with considerable spore production. Spores appear round, dense, opaque. Phase contrast. ($\times 750$.)
- Fig. 3. Strain B₁, growing on cellophan over sterile compost, 1 day old. Long vegetative filament in centre field, showing cluster type of sporulation. Stained by Jones Mollison technique. ($\times 550$.)
- Fig. 4. Strands of branching, segmented, aerial mycelium at margin of colony on CPS agar plate; 2 days old. Phase contrast. ($\times 200$.)
- Fig. 5. Higher magnification of portion of same growth as in Fig. 4, showing cellular contents of aerial filaments and single lateral spores. Spores appear very dense. Phase contrast. ($\times 1800$.)
- Fig. 6. 18 hr. growth produced when a spore suspension in broth was poured along the under-surface of a cover-slip inclined at an angle over CPS agar strips. Note 'scroll' formed by 4-5 filaments elongating in one channel; germinating spore giving rise to 'loop' as direction of new filaments shifts slightly with varying food supply. Arrow marks ungerminated spore. Fixed with osmic acid, stained with Gray's spore stain. ($\times 1500$.) Photograph by Miss Irene Taylor.

(Received 5 December 1952)



D. ERIKSON—REPRODUCTION OF *MICROMONOSPORA VULGARIS*. PLATE 1

ERIKSON, D. & WEBLEY, D. M. (1953). *J. gen. Microbiol.* 8, 455-463.

The Respiration of a Thermophilic Actinomycete, *Micromonospora vulgaris*

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SUMMARY: The respiration of *Micromonospora vulgaris* at 60° was studied by means of the Warburg technique. Various cultural methods and heat treatments were used to separate the different growth phases of this structurally complex organism. One- to two-day growths bearing aerial mycelium were most active. Spores were inactive. Vegetative mycelium which developed within 24 hr. in the Warburg vessels was active, but similar 1-day or older growth harvested from cultures proved to be inactive.

A survey of numerous studies concerned with thermophilic organisms (see Gaughran, 1947) reveals that little attention has been paid to the metabolism of these organisms over the temperature range in which they optimally grow. In his study of certain respiratory enzymes of stenothermophilic bacteria (38-75°), Gaughran (1949) is one of the few who have appreciated the importance of maintaining elevated temperatures throughout an experiment. He performed his experiments over a range of temperatures whose upper limit was 55°. As far as we are aware, no one has attempted to study the respiration of thermophilic actinomycetes. Erikson (1952) isolated one type of thermophilic actinomycete from composts made from fresh lawn cuttings. All the isolates she made were strains of *Micromonospora vulgaris*. No thermophilic streptomycetes were found. The cultural studies over the temperature range of growth emphasized the correlation of a well-developed sporogenous aerial mycelium with the high temperature phase; mesophilic strains of *Micromonospora* sp. do not produce aerial mycelium. In the present work we have attempted to study the respiration of *M. vulgaris* in different mycelial stages at 60°.

METHODS

Isolation and description of strains of Micromonospora vulgaris

Four strains (M, F, D₃ and B₄) isolated from grass composts over successive seasons were used. Their morphological and cultural characteristics are described by Erikson (1953). In addition, a further strain (H) was received from Dr Heller (Ministry of Food, London) who isolated it from canned ham. Apart from a somewhat higher and more consistent heat resistance of the spores, this strain was very like the others.

Technique of growing Micromonospora vulgaris

Composition of medium. A mixture containing tryptic digest of casein (5%, v/v), pea extract (1%, v/v) and soluble starch (1%, w/v) (CPS medium as described by Erikson 1952) was used throughout.

Simultaneous production of top and bottom growth in liquid medium. Large quantities of top and bottom growth were obtained in 50, 100 ml. conical and 500 ml. round-bottomed flasks after incubation for 1–11 days at 60°. Pre-heating of vessels, medium and inoculum at 85° for 5 min., followed by a brief shaking, as previously described (Erikson, 1952), gave the best yields. The method of harvesting top and bottom growth separately is as follows: a short length of glass tubing in the form of a T-piece, having one horizontal arm bent up and the other down, the tail piece being passed just through a rubber bung, is sterilized separately, and fitted into the flask when growth is complete. The flask is then turned upside down, with the result that the non-wetting pellicle of surface growth collects round the walls of the flask while the liquid containing the bottom growth pours through the outlet in the rubber bung directly into sterile centrifuge tubes ready for spinning.

For growth on *solid media*, cellophan circles, either entire or cut into 4–5 strips, were sterilized in the liquid CPS medium, drained, and then placed over poured plates containing CPS medium solidified with agar; the plates were incubated overnight to test for contamination and then sown with a 2-day surface pellicle from a small liquid culture, the growth being evenly distributed over the cellophan with a bent glass rod. Seven or eight plates were stacked in a sterile metal Petri dish container lined with moistened gauze, and incubated at 60°. The maintenance of a humid atmosphere was essential for the production of a good surface growth with abundant aerial mycelium on the cellophan. On the other hand, the presence of excess liquid actually lying on the cellophan tended to prevent the production of aerial mycelium (see Table 3).

Treatment of the material for Warburg experiments

Young growth (1–2 days) with abundant aerial mycelium proved to be most active in respiration experiments.

Liquid medium; surface growth. The material from CPS medium does not suspend uniformly, and in centrifuging the white aerial growth either floated on the top of the suspending medium and/or adhered to the sides of the tubes. The following procedure was more satisfactory. Medium was removed from below the surface pellicle by means of a sterile pipette; as a result the pellicle floated and adhered to the walls of the vessel. To wash this material, sterile distilled water or M/75 phosphate buffer (pH 7.0) was added and again removed from below the pellicle. Finally, a known volume of sterile distilled water, CPS medium or M/75 phosphate buffer, was added and the growth quickly washed into the Warburg vessel from the culture vessel. Later it was found more convenient to lift the washed pellicle from the top of the suspending medium and, with a sterile platinum spatula, to transfer it directly into the Warburg vessel containing the suspending medium. The remaining solutions were then added before the vessels were attached to their manometers.

Liquid medium; bottom growth. Even 1-day growth disintegrates after brief shaking or centrifuging. The material therefore was spun for the shortest time compatible with adequate washing of the cells.

Growth on cellophan strips on solid medium. The strips were curled into the form of a cylinder by using a sterile forceps and the coiled strips bearing the growth put into the Warburg vessels; usually 4–6 coils sufficed. A known volume of sterile distilled water or CPS medium was then added. Usually much of the growth easily detached itself from the strips as they were uncurled and removed aseptically from the vessels. The necessary additions were then made as for liquid cultures. With this procedure there was unavoidable loss of material, as some adhered to the strips as they were removed. Owing to the ease with which vessels became contaminated with thermophilic bacteria, it was necessary before each experiment to sterilize the Warburg vessels, pipettes, solutions, etc., at 22.5 lb./sq.in. for 20 min. At the end of each experiment a nutrient agar plate was streaked from each vessel and then incubated at 60° overnight or longer to test for thermophilic contaminants.

Comparative viable count of samples of material

One unit of each type of material prepared for respiration experiments, e.g. one cellophan strip of *surface growth*, or sample of *surface* or *bottom growth* from liquid culture, was tested the same day to determine the approximate number of viable cells. The difficulties of preparing homogeneous suspensions of mixtures of aerial and vegetative mycelium have already been stressed. Yet with constant practice relative values could be obtained by the methods previously described (Erikson, 1952). Certain minor variations of procedure were introduced in the case of surface growth on liquid media; this material was shaken, with or without glass beads, and filtered through glass wool or ground with flattened glass rods before centrifuging. The growth on cellophan strips suspended more readily, especially after storage in sealed tubes for 18–24 hr. at 0°. The most uniform suspensions were obtained with heated growths. (For details of heating, see Tables 2 and 4.). To economize media 0.1 ml. of each dilution was added to 1 ml. CPS medium + 1 ml. 2.5 % water agar melted in metal-capped 2 oz. bottles and rolled under a stream of cold water. These roll-tubes were incubated in an inverted position within a moistened sterile tin at 60° for 2 days. Counts were obtained which compared reasonably well with the customary poured plates. Thus a rough estimate of the relative number of viable cells used in the Warburg vessels was obtained. The average number of viable units/ml./vessel was 2×10^6 – 10×10^6 from surface growth bearing well-developed aerial mycelium on liquid cultures or cellophan strips. With bottom growth, the numbers of viable units were very variable but always less than from surface growth bearing well-developed aerial mycelium.

Estimation of dry weight of organism used in Warburg experiments. At the end of the experiment, the necks of the Warburg vessels were carefully freed from grease by means of acetone, the KOH papers and solution removed and the central cup cleaned. The vessel + contents (e.g. organism + buffer, etc.) were dried at 106° overnight and then weighed. By subtracting from this value the weight of the vessel + buffer etc., the dry weight of the organism was estimated. Although not an entirely satisfactory method, this proved the only practical way of estimating the dry weight of organism used.

Running the Warburg apparatus at 60°

The apparatus we used has a large deep tank and is fitted with an automatic setting arrangement which allows the heating to be started in the early hours of the morning. It took about 5 hr. to reach 60°. It was occasionally necessary during the experiment to add water, previously heated to the temperature of the bath, to make up for evaporation; this was done between readings. Variations in temperature during an experiment could not be detected on a thermometer graduated to 0.5°. There was however a slight rise or fall in the manometer fluid, amounting at most to 4–5 mm. divisions on the manometer scale. This was due to the interval between the cutting-out of the heating elements and the subsequent attainment of equilibrium, and did not affect the positive results indicated in the tables; small figures which might fall within this range are there noted. It was necessary to use a special sealing grease for the manometer ends and vessel stoppers; Apiezon T Grease (Shell Chemicals Ltd.) was very satisfactory for this purpose, and was easily removed by acetone at the end of experiments. The manometer taps were unaffected by the temperature and no special tap grease was required for them. Occasionally (particularly when the experiments were run for more than 3 hr.) a small column of water (due to condensation of droplets) formed above or below the bend in the manometer arm. This column was usually easy to dislodge by raising and lowering the manometer fluid in the manometer limbs as is done when removing air bubbles in the manometer fluid. Equilibrium time was usually 15 min. and the rate of shaking 90–100 oscillations/min. The volume in each vessel was 2.5 ml. with 0.2 ml. of 5% (w/v) KOH in the centre cup. K_{O_2} was calculated for 60°.

RESULTS

From Table 1 it will be seen that there was a marked oxygen uptake in the presence of CPS medium; control vessels containing sterile CPS medium consistently gave no oxygen uptake. The endogenous respiration in presence of distilled water or phosphate buffer was very small. At the end of experiments some of the organism had usually accumulated on the wall of the vessels while the remainder was not suspended uniformly throughout the liquid. Material adhering to the vessel wall is, however, repeatedly washed during the shaking of the manometers. The shaking broke up the material to a certain extent, but this did not appear to interfere with ability to take up oxygen. This is borne out by the later entries in Table 1. Attempts were made to disperse the growth in the vessels by the addition of non-toxic wetting agents (e.g. Tween 80), but without success. Erikson (1952) also failed to make suspensions of surface growth in this way. Determinations of pH values showed practically no change during the period of the experiment (3 hr.).

The procedure of heating for 15 min. at 85° results in destruction of vegetative mycelium while a considerable proportion of the spores remain viable (Erikson, 1952). Examples of the way in which the oxygen uptake is influenced by this treatment are given in Table 2. A substantial decrease in

Table 1. O_2 uptake of *Micromonospora vulgaris* at 60° in presence and absence of CPS medium

M. vulgaris (strain F) grown for 2 days at 60° on cellophan strips overlying solid CPS medium. Strips either put directly into vessels followed by 2.5 ml. distilled water or CPS medium or M/75 phosphate buffer (pH 7.0) and then removed; or shaken beforehand with a few sterile glass beads in presence of CPS medium or distilled water in wide sterile tubes. Strips removed from tubes and contents pipetted into vessels. Duration of experiment 3 hr.

Suspension medium	O_2 uptake (μ l./hr.; average)	Dry weight of material (mg.)
Undisturbed organisms		
CPS	26.2	1.3
CPS	95.7	3.2
Dist. water	2.4*	5.5
Dist. water	6.4	6.1
M/75 phosphate buffer	9.3	6.4
Organisms shaken with beads		
Dist. water	11.0	nt.
Dist. water	9.8	nt.
CPS	372.4	nt.
CPS	463.0	nt.

* Total O_2 uptake within experimental error (see Methods).
nt. = not tested.

Table 2. Differences in oxygen uptake at 60° of spores and mycelial growth of *Micromonospora vulgaris*

M. vulgaris (strains D₃ and M) grown on liquid CPS medium for 1 day at 60°. Growth then (i) washed once with 2.5 ml. distilled water before placing in Warburg vessels or (ii) heated in distilled water at 85° for 15 min. before placing in the vessels; 0.5 ml. M/15 phosphate buffer + 1.0 ml. CPS medium added with distilled water to give a final volume of 2.5 ml. Strain M also grown for 2 days at 60° on cellophan strips overlying solidified CPS medium. Half of the strips were removed, put into sealed tubes and heated submerged in a water bath at 85° for 15 min. (details, see Erikson, 1952). Strips then put into Warburg vessels containing 1 ml. distilled water. After removal of strips 0.5 ml. M/15 phosphate buffer + 1.0 ml. CPS medium and distilled water added to give a final volume of 2.5 ml. Exps. 1-4, strain D₃ surface growth on liquid medium; Exps. 5-8, strain M, ditto; Exps. 9 and 10, strain M, grown on cellophan.

Exp.	Treatment of organism	O_2 uptake (μ l./hr.; average)	Duration of experiment (hr.)	Dry weight of material (mg.)	Comparative viable count/ml./vessel at beginning of experiment
1	Unheated	63.0	2	2.4	1,750,000
2	Unheated	44.2	2	2.6	nt.
3	Heated	8.4	2	2.1	500,000
4	Heated	4.9	2	2.5	nt.
5	Unheated	85.3	3	1.3	7,900,000
6	Unheated	61.1	3	0.7	nt.
7	Heated	0	3	0.8	850,000
8	Heated	0	3	1.3	nt.
9	Unheated	207.9	1½	3.6	5,000,000
10	Heated	13.5	1½	3.3	700,000

nt. = not tested.

the oxygen uptake followed heating at 85° for 15 min. in all instances. This indicates that the spores have a very low oxygen uptake. All the plates streaked from the vessels containing heated material showed good growth after incubation overnight at 60°, even where no oxygen uptake was observed in 3 hr. (Table 2).

It was repeatedly observed that the more abundant the white aerial mycelium was the more active oxygen uptake this material showed. Experiments with different types of mycelial growth prepared as described in the section on methods emphasize these differences, as can be seen in Table 3.

Table 3. O_2 uptake at 60° shown by different types of mycelial growth of *Micromonospora vulgaris*

M. vulgaris (strains M, B₁ and D₃). Surface and bottom growth (B₁ and D₃) from liquid culture after pre-heating at 85° for 5 min. followed by incubation at 60° for 1–2 days; surface growth removed, and bottom growth spun down. After washing, growth transferred to vessels + 0.5 ml. M/15 phosphate buffer + 1.0 ml. CPS medium + 1.0 ml. distilled water.

Strain M grown on cellophan strips overlying solidified CPS medium for 2 days at 60°. Strips put into Warburg vessels containing 1 ml. distilled water. After removal of strips, 0.5 ml. M/15 phosphate buffer (pH 7.0) + 1 ml. CPS medium added.

Strain	Type of growth	Age (days)	μ l. O_2 uptake (μ l./hr. average)	Duration of experiment (hr.)	Dry wt. of material (mg.)	Comparative viable count/ml./vessel at beginning of experiment
D ₃	Surface; on liquid medium	1	63.0	3	2.4	1,750,000
D ₃	Bottom growth from above	1	0	3	4.0	4,000
M	Surface on cellophan; well-developed aerial mycelium	2	541.2	1½	8.1	10,000,000
M	Surface on cellophan; poor development of aerial mycelium*	2	11.0	1½	2.1	320,000
B ₁	Surface on liquid medium	2	24.8	1½	nt.	8,250,000
B ₁	Bottom growth from above	2	0	1½	nt.	145,000

* The specimen of growth on cellophan which yielded poor aerial mycelium was obtained following excessive moisture on the plates (see Methods).

nt. = not tested.

It is quite clear from these experiments that a *surface growth* which bears abundant aerial mycelium is by far the most active of the various kinds of growth in taking up oxygen under our experimental conditions. From the results of Table 2 we know that only a very small percentage of this oxygen uptake can be due to the spores themselves. This leads to the conclusion that it is the aerial mycelium which is most active. Even here there are differences corresponding to the vigour of the individual culture on specific occasions. It is also clear that dry weight is inadequate as a quantitative measure for the respiration of this type of material, it being impossible to determine for any given sample the relative amounts of spores, aerial and non-aerial mycelium.

When young surface growth of *M. vulgaris* is respiring in the presence of CPS medium in the Warburg vessels, the oxygen uptake consistently increases throughout the course of the experiment. Similarly, we found (Table 4) that old surface growth or young surface growth which had been heated, although showing no immediate uptake of oxygen, did after a lag period begin to respire, and this rate of respiration also increased with time.

The length of the lag period varied according to age, treatment and quantity of material used. Thus in the first four entries in Table 4 the variable lag was also an expression of part of the survivor curve in a heat-test experiment. After 5 min. at 100° it took 3–4 hr. for sufficient spores to germinate and register a measurable amount of oxygen uptake. After 10 min. at 100° no oxygen uptake was evident within 7½ hr. (first day of the experiment), although naturally many spores must have germinated within the period 7–24 hr. in order to give the large oxygen uptake registered the following morning. The much less drastic degree of heating (85° for 15 min.) used as a method of separating spores from mycelium had little damaging effect upon the spores, but in the fifth and sixth entries it will be seen that it took 3–4 hr. for the growth from newly germinated spores to respire at a rate comparable with that of the unheated surface mycelium (2 days old). Older surface mycelial growth (4, 6 and 11 days) seemed very inactive, for the figures shown in Exps. 14, 17 and 9 could be explained as the respiratory rates of the new growth.

Bottom growth from cultures 1, 2, 4 or 11 days old, when transferred to the vessels never showed any oxygen uptake during a 7½ hr. period of observation. When observation was continued for 24 hr., only one of the five (Exp. 16) showed some oxygen uptake. Where positive values for oxygen uptake are given only after a considerable lag, they represent respiration of young (less than 24 hr.) vegetative mycelium which has grown in the Warburg vessels. In normal growth experiments, spores of *M. vulgaris* usually germinate within 1–2 hr. in CPS medium at 60°.

DISCUSSION

A consideration of the results hinges round two points: the validity of the values given by our technique as a measure of respiration at 60°, and the significance of the differences in oxygen uptake observed as between spores, aerial and non-aerial mycelium. In view of the low endogenous respiration of *M. vulgaris* (Table 1), the consistently negative values given by uninoculated CPS medium, and the very high oxygen uptake shown in all positive results, there can be little doubt that the method gives a true picture of the respiration of the organism. We wish to emphasize, however, that our results should be looked upon in a qualitative light. The conventional methods for the study of oxygen uptake by bacteria (e.g. use of homogeneous suspensions) are not suitable for this type of highly organized material. In particular, the expression of quantity of actively respiring material by dry weight lacks precise meaning (e.g. see Table 4, Exps. 17, 18 and 19). It was therefore necessary to depend on ability to obtain different types of growth by suitable cultural

Table 4. *Oxygen uptake of germinating growths of Micromonospora vulgaris in Warburg vessels at 60°*

M. vulgaris (strains H and B₄) grown on cellophan strips overlying solidified CPS medium for 2 days at 60° (H); also surface and bottom growth from liquid CPS medium at 60° after 1, 2, 4, 6 or 11 days (H and B₄). Controls unheated; all other growth heated at 85° for 15 min. In addition, in experiments 2-4, growth on cellophan was heated in sealed tubes for various periods at 100° and then plunged in ice till ready. Strips of cellophan and liquid growth then placed in Warburg vessels followed by 0.5 ml. distilled water; 0.5 ml. m/15 phosphate buffer (pH 7.0) + 1 ml. CPS medium. Shaking was stopped after 7½ hr. and taps were opened. Next day an extra 0.5 ml. of CPS medium was tipped in from side arm, fresh KOH and filter-paper wicks added, and shaking restarted.

Exps. 1-8, strain H, 2-day growth; Exps. 9-12, strain H, 11-day growth; Exps. 13-19, strain B₄ aged (days) in respective experiments: (2) 13; (4) 14; (2) 15; (4) 16; (6) 17; (1) 18; (2) 19.

O ₂ uptake during various intervals											Dry weight of organic material (mg.)	Comparative viable count/ml./vessel at beginning of experiment
Interval (hr.)												
0-1	1-2	2-3	3-4	4-5	6½-7½	23-24						
Exp.	Type of growth	Treatment	O ₂ taken up (μl.)									
1	Cellophan	Control unheated	49.3	124.9	294.1	—	—	—	—	—	7,500,000	
2	Cellophan	Heated, + at 100° for 5 min.	0	0	16.2	—	—	59.4	—	—	231,000	
3	Cellophan	Heated, + at 100° for 10 min.	0	0	0	—	—	0	207.4	—	157,500	
4	Cellophan	Heated, + at 100° for 60 min.	0	0	0	—	—	0	150.4	—	18,000	
5	Surface (liquid)	Unheated	154.8	378.0	619	—	—	—	—	13.9	6,000,000	
6	Surface (liquid)	Heated	0	54.4	62.2	153.0	—	—	—	17.7	2,100,000	
7	Bottom (liquid)	Unheated	0	0	0	0	—	—	0	9.0	262,000	
8	Bottom (liquid)	Heated	0	0	0	0	—	—	0	14.0	20,000	
9	Surface (liquid)	Unheated	0	0	10.0	54.9	—	—	—	—	4,300,000	
10	Surface (liquid)	Heated	0	0	—	29.7	—	—	—	—	3,850,000	
11	Bottom (liquid)	Unheated	0	0	0	0	—	—	0	—	nt.	
12	Bottom (liquid)	Heated	0	0	0	0	—	—	0	—	nt.	
13	Surface (liquid)	Unheated	24.3	—	—	62.9	141.9	—	—	—	3,250,000	
14	Surface (liquid)	Unheated	0	—	—	0	0	—	104.4	—	2,750,000	
15	Bottom (liquid)	Unheated	0	—	—	0	0	—	0	—	145,000	
16	Bottom (liquid)	Unheated	0	—	—	0	0	—	36.0	—	400,000	
17	Surface (liquid)	Unheated	0	0	30.4	47.1	97.5	—	—	2.0	4,450,000	
18	Bottom (liquid)	Unheated	0	0	0	0	0	—	—	17.7	5,000	
19	Bottom (liquid)	Unheated	0	0	0	0	0	—	—	25.4	450,000	

— = not observed.

nt. = not tested.

methods and also on heat treatment as a means of separating vegetative organism from spores. The customary procedure of heating at 85° for 15 min. to destroy vegetative mycelium resulted in a clear and immediate decrease in the oxygen uptake (see Table 2). For the quantities of material used in these experiments, the method is not sensitive enough to measure oxygen uptake by resting spores. Similarly, vegetative mycelium harvested mainly from liquid cultures registered no oxygen uptake even when 1 day old (see Tables 3 and 4). It is probable here that there has been a very considerable drop in respiratory activity after harvesting; the filaments are very fragile and break easily (see Erikson, 1953). Here again the method possibly fails in sensitivity since growth from newly germinated spores which develop in the Warburg vessels themselves was active (see Table 4).

The morphological development so far outlined is analogous to that of thermophilic eubactria. *M. vulgaris*, however, under optimal conditions develops a secondary aerial mycelium of considerable complexity. Table 3 illustrates the differences in oxygen uptake shown by simple vegetative growth and preparations of the organism which have attained their full development. The respiratory activity of this aerial mycelium is of short duration, and diminishes rapidly after 1-2 days, according to experimental conditions.

The senior author (D.E.), who is a member of the scientific staff of the Agricultural Research Council, wishes to thank Prof. J. Cruickshank, for the hospitality of his Department. Grateful acknowledgement is made to the Agricultural Research Council for an expenses grant. We wish to thank Miss Irene Taylor and Miss Mildred Mackay for technical assistance, and Dr G. K. Fraser for his interest throughout the work.

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(Received 5 December 1952)

The Cultivation of *Actinomyces israelii* in a Progressively Less Complex Medium

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SUMMARY: Six strains of *Actinomyces israelii* were trained to grow in continued subculture on a medium composed of 99 volumes of 1 % casein hydrolysate + 1 volume of heart broth, the mixture containing 0.5 % (w/v) glucose. Poor growth of limited viability was obtained in a medium consisting of 1 % casein hydrolysate + 0.5 % glucose (w/v) only. The addition of a wide variety of different amino-acids, fatty acids, growth factors and mineral salts to certain of the less complex media did not compensate for the partial or complete omission of the heart broth. Of the glucose supplied in relatively complex media about 40 % was utilized, and lactic acid equivalent to 30-60 % of the glucose utilized appeared in the medium.

Actinomyces israelii, the anaerobic organism which is the causal agent of actinomycosis in man and in some animals, has a well-established reputation for being difficult to maintain in laboratory culture. Alternation of media such as cooked meat + serum, nutrient agar + sterile horse blood, concentrated sheep heart broth + glucose, has been recommended (Erikson, 1940). Rosebury (1944) noted the advisability of a 'mixed diet', and found brain + heart infusion of especial value. Later workers (e.g. Thompson, 1950; Holm, 1950) used brain broth, 'hormone' agar, blood agar or broth supplemented with ascitic fluid. Brewer's (1940) thioglycollate (mercaptoacetate) medium, in common use for this and other anaerobes, contains pork infusion solids. Such media are amongst the richest used for the cultivation of micro-organisms, yet short life, frequent dying out and at best lessened activity attend the prolonged use of any one of these complex media for the cultivation of *A. israelii*. The following report describes an attempt to produce simpler media, and the growth characteristics obtained thereon.

METHODS

Organisms. Five human strains, typical *Actinomyces israelii*, and one porcine strain closely resembling these, were selected for detailed study. All six strains had been freshly isolated from cases of actinomycosis.

Apparatus. Except where otherwise mentioned all liquid media were dispensed into rimless test-tubes 12 × 75 mm., plugged with cotton-wool.

Automatic siphon-burettes of 2 or 10 ml. capacity which could be sterilized and also dismantled for cleaning were devised to fit 500 ml. (20 oz.) screw-capped 'medicine flats' and 25 ml. screw-capped bottles. These burettes were used for dispensing all media.

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All new glassware was given a preliminary cleansing in hot chromic + sulphuric acid mixture. For routine cleansing glassware was, wherever possible, boiled with soap and water, scrubbed with hot soapy water, rinsed with tap water, then with distilled water and finally dried in the oven. Apparatus not amenable to this treatment was cleaned in hot chromic + sulphuric acid mixture, drained, rinsed successively in tap water, dilute NaOH, tap water, distilled water, and then dried. Before contact with any medium all apparatus was sterilized by autoclaving at 20 lb./sq.in. for not less than 30 min., and thereafter dried in the oven.

All measurements of pH values were made electrometrically using a glass electrode.

Anaerobiosis. Culture tubes were stacked in two layers with an intervening wad of filter-papers in standard anaerobic jars. Jars were evacuated to 1 cm. Hg and filled with commercial compressed nitrogen (c. 99.8 % pure) which had been passed through three alkaline pyrogallol towers. This procedure was repeated twice.

Chemicals. D-Glucose (anhydrous A.R., British Drug Houses Ltd.); casein hydrolysate, 'Vitamin free for microbiological assay' (Allen and Hanbury); casein, light white soluble (British Drug Houses Ltd.); agar, New Zealand (Davis Gelatin Co. Ltd.); 2-amino-2-(hydroxymethyl)-1,3-propanediol (Eastman-Kodak); KH_2PO_4 (A.R., British Drug Houses Ltd.); NaOH (A.R.). All other inorganic chemicals mentioned in the text were of 'reagent' quality; other organic chemicals mentioned were the best available and were not further purified.

Media

The six strains of *A. israelii* have been carried in repeated subculture in each of several media for varying periods. The composition of these media is given in Tables 1 and 2.

Table 1. *The composition of liquid media for the maintenance of Actinomyces israelii in continuous subcultivation*

Components	Media			
	S	A ₁	A ₂	A ₃
Heart broth	1 volume	1 volume	1 volume	1 volume
Casein tryptic digest	1 volume	—	—	—
Casein hydrolysate (1 %, w/v)	—	1 volume	1 volume	1 volume
Glucose	0.5 % (w/v)	0.5 % (w/v)	—	—
Glycerol	—	—	0.5 % (w/v)	—
Starch	—	—	—	0.04 % (w/v)

Table 2. *The composition of the dilution media A-O used for training Actinomyces israelii to grow in progressively less complex media*

Glucose at 0.5 % (w/v) was added to each medium.

Components	Dilution media										
	A	B	C	D	E	F	G	K	L	M	O
Heart broth (% v/v)	50	40	30	20	10	4	2	1	0.5	0.25	0.0
Casein hydrolysate, 1 % solution (% v/v)	50	60	70	80	90	96	98	99	99.5	99.75	100.0

Preparation of components of media

Heart broth. Beef hearts were used according to the method of Wright (Mackie & McCartney, 1938) with the following modifications: addition of NaCl, peptone and glucose was omitted; initial hot extraction of the mince was accompanied by vigorous mechanical stirring; subsequent steaming was performed in small batches (not more than 500 g. mince and 1 l. distilled water); filtration was through a closely packed wad of glass wool surmounted by the extracted meat. The final water-clear filtrate was adjusted by addition of N-NaOH to pH 7.2.

Casein digest. Casein (200 g.) was homogenized with distilled water (2 l.) in an Atomix (Measuring and Scientific Equipment Ltd., 14/28 Spenser St, London, S.W.1) homogenizer. The suspension obtained was adjusted to pH 8.2 by addition of 10N-NaOH. The preparation otherwise proceeded according to Cole & Onslow (1916). The final digest was adjusted to pH 7.2.

Casein hydrolysate solution. Casein hydrolysate powder (5 g.) dissolved in distilled water (450 ml.) was adjusted by addition of N-NaOH to pH 7.2. The water-clear solution was made to 500 ml. with distilled water.

Sterilization of components of media. The heart broth, casein digest and casein hydrolysate solutions were separately placed in 500 ml. quantities in sterile screw-capped 'medicine flats' (20 oz.) and sterilized by autoclaving for not more than 10 min. at 20 lb./sq.in. The two casein media invariably remained water-clear. In the few instances where the heart broth did not stay clear it was centrifuged, the supernatant solution removed by decantation and reesterilized as before, when it remained clear.

Mixing and dispensing of complete media. Media S, A₁, A₂ and A₃ (Table 1) were prepared in 500 ml. volumes as required by mixing aseptically their respective sterile components in sterile screw-capped bottles which were then steamed for not more than 5 min.; this steaming was repeated each time a bottle was opened. These media were dispensed aseptically in 2.5 ml. amounts via sterile burettes into sterile tubes which were then steamed for not more than 5 min. In every case a water-clear medium was obtained which had little or no tendency to give a precipitate on storage or incubation, except where the pH value decreased during growth. The amount of growth in these media was far superior to that in media of the same initial composition but which had been autoclaved after mixing the constituents. Repeated steaming of the completed media caused a slight diminution in growth. A sufficient volume of medium for immediate use was therefore made up at one time. Between 200 and 400 tubes of medium have been dispensed in this way each week during the past two years. The number of contaminated tubes had been negligible.

The dilution media A to O (Table 2) were compounded by aseptic dispensing of each sterile component solution in proper proportion via sterile burettes into sterile culture tubes (final volume of medium, 2.6 ml.) which were then steamed for not more than 5 min. Thus the completed media were steamed only once before inoculation; they were superior to media of the same initial

composition prepared as described for media S, A₁, A₂, and A₃. The tubes of dilution media remained water-clear, except when growth of the organism caused a marked decrease in pH value, and were practically colourless. This dispensing technique was too slow for routine work. Fifty to 100 tubes/week have been dispensed in this way over the last 18 months with a negligible number of contaminated tubes.

Methods of cultivation

Stock cultures were carried in the liquid media described above. Transfers were made by Pasteur pipette at intervals of not more than 7 days. The inhomogeneity of growth of the organisms made it impossible to use a measured volume of cell suspension as inoculum. Accordingly, three or four small (c. 1 mm. diam.) colonies or fragments of colonies were used as inoculum in transfers to fresh medium.

In the initial work organisms washed with sterile 0.85% NaCl before subculture appeared to multiply more rapidly than unwashed organisms; later results were inconsistent, and an improvement in growth was again obtained by brief washing with sterile distilled water. It subsequently became clear that the frequency of subcultivation was the important factor; the inoculum is not now washed.

Shake and slope cultures on nutrient media (equal volumes of heart broth and casein digest without carbohydrate) were inoculated at each subculture; after maximum growth was attained the tubes were sealed with paraffin wax and stored at 2°. These cultures were used as additional checks on the purity of cultures, and, when necessary, as a source of renewed subcultures. The incubation temperature was 37°.

RESULTS

Two strains (X and Y) of the six selected for study had originally been cultivated for several months in various complex media prepared by the more usual techniques. When repeatedly subcultured in medium S (Table 1) growth was at first slight and it was only possible to maintain the cultures by inoculating large numbers of tubes of medium (liquid, slopes and shakes). Subcultivation was necessary at intervals of not more than 7 days. Originally the liquid medium was dispensed in 10 ml. amounts in 18 × 150 mm. tubes. To conserve medium and to obtain a larger number of inoculated tubes, the smaller tubes mentioned previously (under *Apparatus*) were adopted. Much better mass of growth, in proportion to the volume of medium, then resulted, presumably due to more rapid attainment of equilibrium conditions between gas and liquid phases. Good growth in medium S which was continuously reproducible on subcultivation, was only obtained after 7–15 subcultures with strains X and Y and with other strains tested which had received similar initial treatment. The remaining four strains were taken into medium S immediately after isolation in pure culture; good growth, continuously reproducible on subcultivation in medium S, was obtained after three or four subcultures. Medium S was therefore particularly suitable for recently isolated

strains; patience and persistence were required to bring old stock cultures into a reliable vigorous state of growth.

Paper chromatographic analysis (Consden, Gordon & Martin, 1944) of the amino-acid content of the casein digest and the casein hydrolysate suggested that replacement of the casein digest in medium S by the casein hydrolysate should be the first step towards the attainment of a defined medium. The casein digest and casein hydrolysate contained approximately the same proportions of those amino-acids which were present in both. The casein digest lacked proline and hydroxyproline and contained only traces of glycine and aspartic acid; the casein hydrolysate lacked tryptophan and contained only traces of hydroxyproline and cystine. Accordingly media A_1 , A_2 and A_3 (Table 1) were prepared. As the next step the heart broth was progressively eliminated from medium A_1 and replaced by similar volumes of casein hydrolysate (1%, w/v) to give the series of dilution media A-O shown in Table 2. The cultural characteristics of the six selected strains of *A. israelii* in any one of the media listed in Tables 1 and 2 were remarkably similar. In what follows these cultural characteristics are described.

Cultures in media A_1 , A_2 , A_3 and S

All strains were grown initially in medium S. It has been possible in every case to maintain healthy growth in this medium for an apparently indefinite period by weekly transfers of fresh medium; one strain has been in continuous cultivation in this medium for one year, the other five for periods up to two years. Once good reproducible growth was obtained in medium S transfers were made to media A_1 , A_2 and A_3 and four parallel cultures are now maintained. Growth in medium A_1 has always been good and it has been possible to maintain continuous cultures for 18 months by weekly transfers. Growth has not, however, been as prolific as in medium S (Pl. 1, fig. 1 and Table 3). In media A_2 and A_3 repeated subcultures maintained in the same way tended to decrease in vigour during the first ten weeks. Subsequent cultivation during 6 to 9 months has shown no diminution in vigour of the cultures.

In media A_1 , A_2 and S all strains grew as discrete, compact, medium-sized 'bread-crumbs', 'cauliflower' or 'puffball' colonies which settled in the bottom of the culture tubes. Clouding of the medium did not take place. In medium A_3 the colony form was frequently the same as in media A_1 , A_2 and S but the colonies were almost invariably accompanied by much greater numbers of minute puffball colonies, even in cases where, as in one of the glucose media, it could be claimed that abnormally large cauliflower colonies were typical; occasionally only minute colonies appeared in medium A_3 . Apart from colony form, growth in this (0.04%) starch medium was usually accompanied by a slight but distinct opalescent haze in the immediate vicinity of the colonies, the medium as a whole remaining clear.

In medium S the pH value fell as low as 4.6 during 7 days growth; the medium remained water-clear. In medium A_1 the pH value fell as low as in medium S, but below pH 5.2 a flocculent precipitate usually appeared. In medium A_2 the pH value decreased only slightly during growth; the maximum

decrease recorded was from pH 7.2 to 5.5 during 7 days. The more usual terminal pH value was 6.5; the medium remained clear. In medium A₃ the usual terminal pH value was 6.5.

Better growth was obtained by buffering medium A₁. The casein hydrolysate moiety of this medium was compounded in Sorensen's M/15 phosphate buffer, pH 7.2 (Clark, 1928) instead of in distilled water, with adjustment of pH value and sterilization as before. Similarly, a casein hydrolysate solution (1%, w/v) containing M/15 2-amino-2-(hydroxymethyl)-1,3-propanediol was prepared and adjusted to pH 7.2. Medium A₁ which contained either of these buffered casein hydrolysate solutions gave amounts of growth which approached that obtained in medium S (Pl. 1, figs. 1, 2; see also Table 3).

Cultures in dilution media A-D

Serial cultivation of strains X and Y were made: from medium A₁→to dilution medium A→B→C→D. In the first few subcultures growth was only obtained in media A and B. Twelve subcultures were necessary before minimal growth was obtained in medium D; another fifteen subcultures were necessary to train the organism to grow in medium F. With the four freshly isolated strains growth in medium F was established in half the time. By proceeding in this manner the proportion of heart broth in the medium was progressively decreased (Table 2) until five of the six strains were trained to grow consistently on medium K (heart broth, 1 vol.; 1% casein hydrolysate, 99 vol.; glucose, 0.5% w/v). The exceptional strain has grown well only in dilution media A-D. Some growth was obtained with all strains in all dilution media but below the limits mentioned growth was slight and of limited viability. Growth in the series of dilution media occurred in graded amounts; with the inocula mentioned, the amount of growth in medium K (medium D in the case of the exceptional strain) was sufficient to provide viable inocula for twelve fresh tubes of medium (see Pl. 1, fig. 3, and Table 3).

Cultures in supplemented dilution media D and F

When reproducible growth had been established in media D and F the effect of adding defined compounds to these media was tried. All supplements were adjusted to pH 7.2, made to volume and sterile 0.1 ml. samples added aseptically to the media. Except where otherwise stated no consistent enhancement or inhibition of growth was obtained in repeated trials during 6 to 12 consecutive subcultures with any of the following compounds at the concentrations stated:

Thiamin, riboflavin, nicotinic acid, nicotinamide, pyridoxine, pantothenic acid, *p*-aminobenzoic acid, inositol, choline, ascorbic acid (each 10 µg./ml.), folic acid (0.4 mµg./ml.), biotin (80 mµg./ml.), vitamin B₁₂ (1 µg./ml.), tested singly or together.

Glycine, DL-alanine, DL-serine, DL-threonine, DL-valine, DL-norvaline, DL-leucine, DL-isoleucine, DL-norleucine, DL-aspartic acid, DL-glutamic acid, L-asparagine, DL-glutamine, L-arginine, L-lysine, L-histidine, DL-ornithine, DL-citrulline, DL-methionine, L-phenylalanine, L-tyrosine, DL-iodotyrosine,

DL-tryptophan, L-proline, sarcosine (100 $\mu\text{g./ml.}$) tested individually; the addition, singly or together, of L-cystine, L-hydroxyproline and DL-tryptophan at the same concentrations to medium O had no effect.

Table 3. *Typical yields of Actinomyces israelii harvested after 7 days anaerobic growth in the various media shown*

Media A₁, A₂, A₃, S and A-O: see Tables 1 and 2.

Medium a: medium A₁ containing M/15 2-amino-2-(hydroxymethyl)-1,3-propanediol, pH 7.2.

Medium b: medium A₁ containing M/15 phosphate buffer, pH 6.0.

Medium c: medium A₁ containing M/15 phosphate buffer, pH 7.2.

Medium d: medium A₁, pH 7.2—no buffer added.

Wet weights determined after washing cells three times with distilled water; dry weights determined after drying over H₂SO₄ *in vacuo*; total N by Kjeldahl digestion.

	Stock media				Buffered media			
	A ₁	A ₂	A ₃	S	a	b	c	d
Wet weight (mg./10 ml. medium):	29.1	10.3	30.6	79.9	60.0	20.0	55.6	37.6
Dry weight (mg./10 ml. medium):	1.7	0.5	1.4	3.9	2.8	—	2.8	2.0
Total N ($\mu\text{g./10 ml. medium}$):	166	53	118	283	252	52	268	152
Wet weight } Dry weight }	17.1	20.6	21.8	20.5	21.4	—	19.9	18.8
Dry weight } Total N }	10.2	9.4	11.0	13.8	11.1	—	10.4	13.1

	Dilution media										
	A	B	C	D	E	F	G	K	L	M	O
Wet weight (mg./10 ml. medium):	30.9	35.8	27.6	31.1	22.8	7.8	2.8	6.2	1.3	0.4	0.0
Dry weight (mg./10 ml. medium):	2.2	2.3	1.7	2.0	1.8	0.5	0.2	0.4	0.1	0.0	0.0
Total N ($\mu\text{g./10 ml. medium}$):	223	194	147	154	110	41	15	26	5	—	—
Wet weight } Dry weight }	14.0	15.6	16.2	15.5	12.7	15.6	14.0	15.5	13.0	—	—
Dry weight } Total N }	9.8	11.8	11.5	13.0	16.3	12.2	13.3	15.4	20.0	—	—

Acetic, propionic, butyric, valeric, caprylic, capric, lauric, myristic, palmitic, stearic, oleic, linoleic, undecylic, traumatic, behenic acids (10, 100 and 1000 $\mu\text{g./ml.}$), tested individually; acids with molecular weights greater than that of caprylic acid tended to inhibit growth at 1000 $\mu\text{g./ml.}$; malonic, succinic, malic, fumaric, citric acids (0.01 M).

Thymine, uracil, adenine, guanine, xanthine (each 100 $\mu\text{g./ml.}$), creatine, creatinine, cholesterol, taurocholic acid (100 $\mu\text{g./ml.}$).

Chlorides of Na, K, Ca, and Mg were added singly, in certain pairs (Na + K) and (Ca + Mg), and all four combined to give final concentrations simulating those in whole blood, serum, blood cells, or muscle; supplements of the following salts: KI, (NH₄)₂SO₄, MgSO₄, FeSO₄, KClO₃, (0.01 M).

Sodium fluoride (0.01 M) and NaCN (> 0.0002 M) inhibited growth.

Longevity of cultures in the presence of various carbohydrates

The basal medium chosen for fermentation tests and for longevity tests in presence of various carbohydrates was heart broth (1 vol.) + casein digest (1 vol.), with the addition of 1 ml. Andrade's indicator/100 ml. complete medium. Each carbohydrate was added to a final concentration of 0.5 % (w/v) except starch which was added to final concentration 0.04 % (w/v).

Table 4. *The relative amounts of growth, and of acid production, and relative longevity of cells in cultures of Actinomyces israelii under various conditions*

Cultures were subcultivated at weekly intervals and also kept in continuous incubation in unchanged media in the presence of various carbohydrates.

Supplement	Relative amount of growth	Relative acid production	Longevity		
			14 days	21 days	35 days
Glucose	+++	AAA	LLL	LL	L
Fructose	+++	AAA	LLL	L	0
Sucrose	+++	AAA	LLL	L	L
Maltose	+++	AAA	LLL	0	0
Raffinose	+++	AAA	LLL	L	L
Xylose	++	AA	LLL	0	0
Rhamnose	++	AA	LLL	LLL	LL
Glycerol	+++	0	LLL	LLL	LLL
Sorbitol	+	0	LLL	LLL	LLL
Mannitol	+	0	LLL	LLL	LLL
Starch	+++	0	LLL	LLL	LLL
Dextrin	+++	0	LLL	LLL	LLL
Glycogen	+++	0	LLL	LLL	LLL
Nil	+++	0	LLL	LLL	LLL

+++ Excellent growth in 7 days, increasing markedly during 35 days.

++ Fair growth in 7 days, increasing slightly during 35 days.

+ Slow growth in 7 days, increasing very slightly during 35 days.

AAA rapid acid production in 7 days, continuing over 35 days.

AA slow acid production in 7 days, increasing slightly in 35 days.

Viability: LLL excellent, LL fair, L poor viability after successive weekly subcultures and under continuous incubation in unchanged medium at the times noted.

Strains X and Y were used and inoculations made initially from medium S. The tubes so inoculated were incubated continuously except for brief examination at weekly intervals for growth and acid production. At the end of each week of incubation, small inocula were transferred to fresh tubes of medium S to test viability. At the end of the first week of incubation of the initially inoculated tubes of carbohydrate media, inocula were removed to fresh tubes of similar carbohydrate media; at the end of a further week these last cultures were again transferred to fresh carbohydrate media and repeated serial subcultures maintained in this way. The results (Table 4) showed that the rate and quantity of growth roughly paralleled the rate and quantity of acid production; conversely the organism remained viable longest in those media containing supplements from which least acid was produced.

Glucose utilization and acid production during cultivation

Cells of all six strains were collected after the usual period of cultivation in media S, A₁, A₂, A₃ and dilution media F and G (Tables 1 and 2). The corresponding culture filtrates were analysed for glucose (method of Nelson, 1944) and for lactic acid (method of Barker & Summerson, 1941). Uninoculated tubes incubated under the same conditions served as controls. The results showed that of the glucose supplied *c.* 40% was utilized and lactic acid equivalent to *c.* 30–60% of the glucose utilized appeared in the medium. Lactic acid was not formed in the glycerol medium (A₂) or in the starch medium (A₃).

The effect of reducing substances on growth

Sodium mercaptoacetate, benzenesulphinic acid or mercaptoethanol at final concentrations from 0.1 to 2.5% were added to medium S. Under anaerobic conditions the addition of these substances at 0.1% made no difference; under aerobic conditions only very slight growth occurred as thin wispy colonies of limited viability. Under aerobic or anaerobic conditions these substances at 1.0–2.5% were toxic. The addition of glutathione or cysteine under anaerobic conditions had no effect.

DISCUSSION

In earlier work the complex media used may have been beneficial in improving the reducing conditions, thereby counteracting, in part at any rate, poor anaerobic techniques. On the other hand such rich media may contain substances which are inhibitory to growth as well as those which are necessary for the organism.

In the present work we used an anaerobic technique which involved repeated evacuation and refilling of a vessel containing the culture tubes with nitrogen gas which has been passed through alkaline pyrogallol. This ensured removal of oxygen from the gas phase and also a considerable loss of dissolved oxygen from the fluid phase.

This enabled adequate anaerobic conditions to be established by an easy, repeatable technique and considerable simplification of the culture medium did not prove impossible.

Continuous subcultivation in the media described appears to depend on: separate sterilization of the components of media; sterilization under adequate but milder-than-usual conditions in previously sterilized containers; aseptic dispensing of the sterile components of the media; the use of the simplest possible medium, *e.g.* casein digest or hydrolysate with minimal supplements of broth + glucose, glycerol or starch; frequent subcultivation; good anaerobic conditions during cultivation. Our technique of progressive elimination of the heart broth moiety of a broth + casein hydrolysate + glucose medium has undoubtedly been justified, but we have failed to obtain a completely defined medium for *A. israelii*. Repeated attempts to eliminate the broth completely yielded organisms of only very limited viability.

The growth of our strains of *A. israelii* in each of our media during two years has remained typical: in liquid culture colonies were discrete, compact, hard, 'bread-crumbs', 'cauliflowers' or 'puffballs' which remained at the bottom of the culture tubes, the rest of the medium remaining clear. Surface colonies on solid media were abruptly raised, nodular, and appeared wet and glistening. No softening of the colonies, sudden increases in amounts of growth, development of an aerophilic or micro-aerophilic phase, or pigmentation of colonies has been observed in pure cultures; such changes we regard as indicative of contamination. The microscopic appearance of the cells remained characteristic throughout. It appears therefore that *A. israelii*, if treated according to our techniques soon after isolation in pure culture, is not difficult to maintain in culture for protracted periods.

We wish to thank: the Medical Research Council for an expenses grant and a personal grant to one of us (J. W. P.); Prof. J. Cruickshank, Department of Bacteriology, and Prof. W. O. Kermack, F.R.S., Department of Biological Chemistry, for the hospitality of their respective departments; Miss Helen Stewart, for technical assistance; Dr R. W. Riddell, Dr J. Walker and Prof. L. P. Garrod for cultures from human pathological material; Mr W. MacDonald of the Aberdeen Flesher Incorporation for supplies of beef hearts; Mr P. Bruce of Robert Lawson and Sons Ltd., Dyce, Aberdeenshire, for supplies of pig pancreas; Glaxo Laboratories Ltd. for crystalline vitamin B₁₂, obtained through the courtesy of Dr T. F. Macrae; Mrs M. Gerrie for help with the preparation of the typescript.

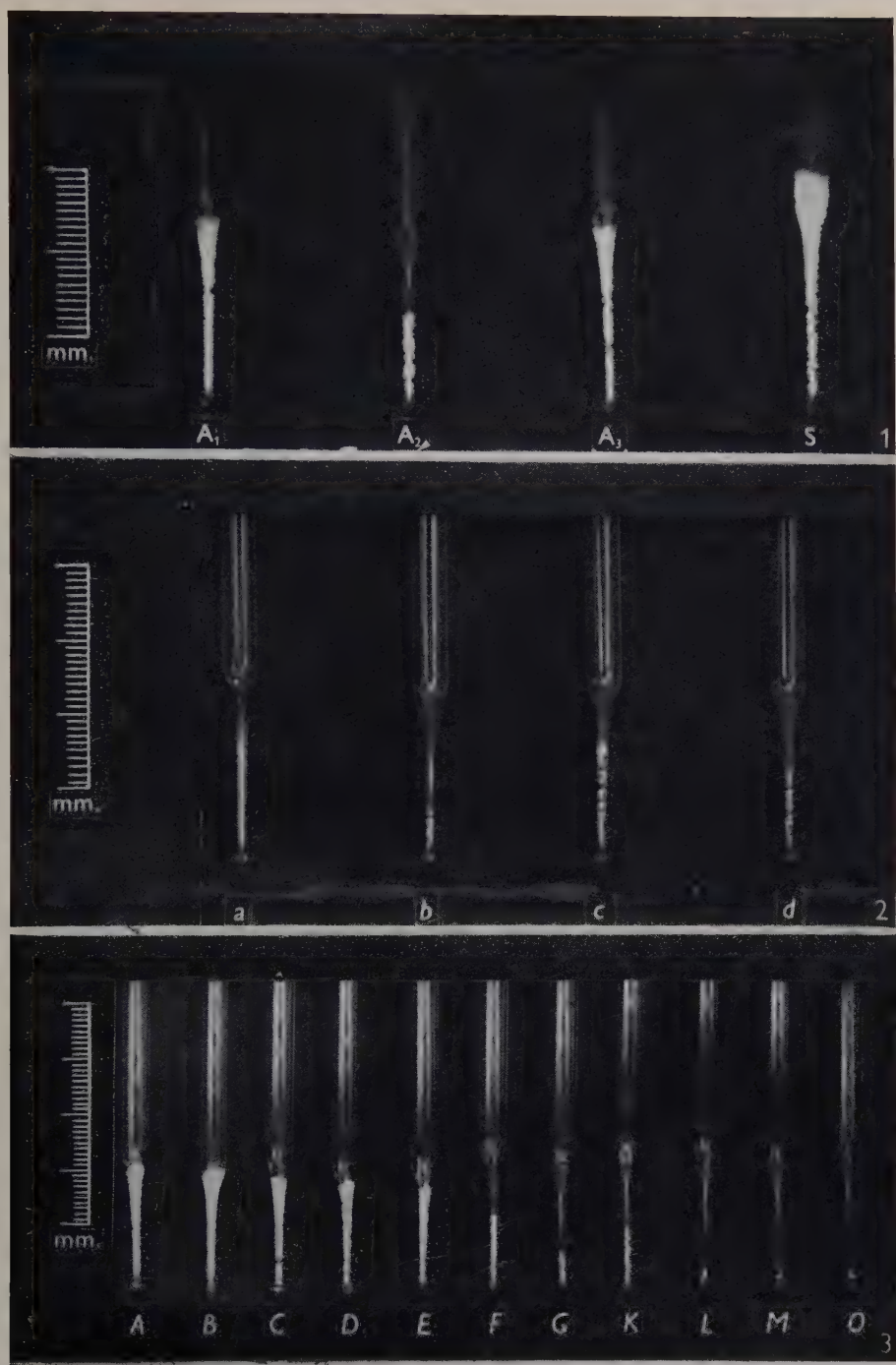
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EXPLANATION OF PLATE

- Fig. 1. Cells of *Actinomyces israelii* harvested into microcentrifuge tubes after anaerobic growth for 7 days in media A₁, A₂, A₃ and S (Table 1). Four culture tubes containing a total of 10 ml. medium were harvested in each case.
- Fig. 2. Cells of *Actinomyces israelii* harvested into microcentrifuge tubes after anaerobic growth for 7 days in buffered and unbuffered medium A₁ (Table 1 and p. 469): (a) buffered with m/15 2-amino-2-(hydroxymethyl)-1,3-propanediol, pH 7.2; (b) buffered with m/15 phosphate buffer, pH 6.0; (c) buffered with m/15 phosphate buffer, pH 7.2; (d) no buffer added. One culture tube containing 2.5 ml. medium was harvested in each case.
- Fig. 3. Cells of *Actinomyces israelii* harvested into microcentrifuge tubes after anaerobic growth for 7 days in media A-O (Table 2). Four culture tubes containing a total of 10 ml. medium were harvested in each case.

(Received 5 December 1952)



D. ERIKSON & J. W. PORTEOUS—THE CULTIVATION OF *ACTINOMYCES ISRAELII*. PLATE 1

Aerococcus, a New Bacterial Genus

By R. E. O. WILLIAMS, ANN HIRCH AND S. T. COWAN

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SUMMARY: The generic name *Aerococcus* is proposed for a group of aerobic Gram-positive cocci that are commonly found in the air of occupied places and in dust. The most characteristic features of the organisms are ability to grow in the presence of 40 % bile and to produce greening on blood agar. They are catalase-negative, and do not show chain-formation in culture.

For the collection and recognition of mouth streptococci from air a selective culture medium was devised (Williams & Hirsch, 1950) which had the composition: serum agar, 100 ml.; sucrose, 5 g.; potassium tellurite, 1.0 mg.; crystal violet, 0.25 mg. This selective medium inhibits the growth of diphtheroid and coliform organisms and practically all strains of those Gram-positive cocci that would ordinarily be regarded as staphylococci. It permits good growth of most streptococci, including *Streptococcus pyogenes*, viridans-type streptococci and enterococci. However, in air samples from occupied rooms, about 70 % of the colonies are not of typical streptococci, but are of a Gram-positive coccus that does not appear to have been adequately described in the literature. We consider that this organism has sufficient points of difference from both streptococci and staphylococci to justify its being placed in a new genus for which we propose the name *Aerococcus*.

DESCRIPTION OF *AEROCOCCUS*

Cultural and morphological characteristics

After 18-24 hr. incubation at 37° on blood agar the colonies of *Aerococcus* are 0.5-2.0 mm. in diameter, semi-transparent, white or grey, and surrounded by a wide or narrow zone of greening (Pl. 1, fig. 1); with some strains the centre of the area of greening may be very dark. The organisms grow well on nutrient agar with or without 0.5 % glucose, and on serum agar, chocolate agar and blood agar. The colonies are larger on blood agar than on the other media; the addition of serum or glucose to nutrient agar results in only slight increase in colony size. Growth is not enhanced, and may be slightly decreased, by incubation anaerobically or in air with 30 % carbon dioxide. The green discoloration round colonies on blood agar or chocolate agar is much decreased in depth and area when the cultures are incubated anaerobically. On all these media growth takes place at 22°, the colonies taking about 42 hr. to reach the size reached in 18 hr. at 37°. There is no growth on potato. In nutrient broth or peptone water growth is very poor, but it is considerably increased by the addition of 0.5 % glucose.

Microscopically the organisms are round Gram-positive cocci 1-2 μ . in

diam., usually staining deeply, arranged in pairs or irregular clusters (Pl. 1, fig. 2); different strains vary considerably in the size of the cells and in their arrangement. Chains are not formed on solid or in fluid media and the paired cocci do not show elongation along the axis joining their centres, as is seen with pneumococci.

The biochemical activities of twelve representative strains of these organisms are given in Table 1.

Table 1. *Cultural characteristics of aerococci*

	<i>Aerococcus</i> <i>viridans</i> : NCTC 8251	No. positive of 12 strains tested
Greening on blood agar	+	12
Growth in the presence of 40 % bile	+	12
Growth in the presence of 1/2500 potassium tellurite on the medium of Anderson <i>et al.</i> (1931)	—	0
Growth at pH 9.6	+	11
Growth at 45°	—	0
Resistance to 60° for 30 min.	+	12
Reduction of 0.1 % methylene blue in milk	—	0
Acid but no clot in litmus milk	+	12
Liquefaction of gelatin	—	0
Hydrolysis of: Arginine	—	0
Aesculin (in solid medium)*	±	12
Starch 1 % (in solid medium)	—	0
Acid produced from: Glucose	+	12
Maltose	+	12
Lactose	+	10
Mannitol	+	5
Sucrose	+	12
Raffinose	—	4
Final pH value in 1 % glucose broth	5.5–5.8	12
Catalase production	—	0
Coagulase production	—	0

* 0.10 % aesculin was incorporated in the 40 % bile agar with 0.05 % ferric citrate as indicator. After 24 hr. incubation the medium was unchanged, but after another day on the bench some hydrolysis of the aesculin, indicated by blackening of the plate, had occurred.

Acid and formamide extracts of many strains have been tested for precipitinogen reacting with antisera to Lancefield Group D streptococci but none has been found, even after ethanol precipitation (Shattock, 1949). An attempt was made to prepare an antiserum against one typical strain but it did not react with extracts of the vaccine strain, other aerococci, or enterococci. The organisms tend to give rather granular suspensions in saline or broth; none of six strains giving smooth suspensions was agglutinated by antisera to various Lancefield groups of streptococci or to *Staphylococcus aureus*.

In many respects the organisms resemble streptococci, particularly enterococci (see Table 2). Thus they either fail to produce catalase or produce only the merest trace of it; they are benzidine-positive; they flourish on serum agar containing 40 % bile and on agar containing 1/400,000 crystal violet and 1/100,000 potassium tellurite.

On the other hand, microscopically the organisms do not resemble streptococci. Although it is true that some strains of undoubted streptococci show few or no chains, we do not think that one could ever examine preparations from several thousand streptococci without seeing any with definite chain formation. But this is the case with the aerococci.

Table 2. *Comparison of biochemical reactions of Aerococcus viridans with those of Streptococcus faecalis and Str. bovis*

	<i>Aerococcus viridans</i>	<i>Str. faecalis</i> and variants	<i>Str. bovis</i>
Growth on 40 % bile	+	+	+
Resistance to 60° for 30 min.	+	+	—
Growth at pH 9.6	+	+	—
Growth at 45°	—	+	+
Growth on tellurite medium	—	±	—
Hydrolysis of arginine	—	+	—
Reduction of 0.10 % methylene blue in milk	—	+	—
Litmus milk	Slight acid	Reduction	Acid
Final pH value in 1 % glucose broth	5.5–5.8	4.0–4.8	4.0–4.8

Shaw, Stitt & Cowan (1951) have already pointed out that the aerococci, which they referred to as their 'α-group', seem to be distinguished from the staphylococci by the absence or extreme poverty of catalase production, by the fact that the colonies are semi-transparent, and by the fact that on blood agar they are surrounded by a definite zone of green coloration. We consider, therefore, that they constitute a distinct genus in many ways intermediate between *Staphylococcus* and *Streptococcus*; and from the source in which we first observed them we propose as the generic name, *Aerococcus*. The genus is probably more closely related to *Streptococcus* than to *Staphylococcus*, and should be placed in the same family as *Streptococcus*.

The genus is defined as follows:

Aerococcus. *Constant characteristics*. Gram-positive, non-motile cocci occurring in pairs or irregular clusters, usually small. Aerobic and facultatively anaerobic; growth occurs on solid media at 22° almost as well as at 37°, but not at 45°. Colonies on blood agar incubated aerobically at 37° for 18–24 hr. are semi-transparent and surrounded by a zone of green colour. Growth is not inhibited by 40 % bile, nor by 1/400,000 crystal violet. Arginine is not hydrolysed. The organisms survive heating to 60° for 30 min. and grow at pH 9.6. Acid and formamide extracts do not react with sera prepared against Lancefield Group D streptococci.

Variable characteristics. Table 1 indicates that some of the carbohydrate-fermentation activities vary from strain to strain. A representative strain isolated from the air of an occupied room has been deposited in the National Collection of Type Cultures as the type strain (NCTC 8251), with the name *Aerococcus viridans* n.sp. Its characteristics are included separately in Table 1.

Ecology

Our attention was first drawn to these organisms by the fact that they confused our search for air-borne viridans streptococci; they constituted the great majority of the colonies on plates of the crystal-violet potassium tellurite medium that was employed. The following figures indicate their general prevalence in the air of occupied places, as determined by counting plates exposed in a slit-sampler (Bourdillon, Lidwell & Thomas, 1941):

	Colonies per cu.ft. air		No. of observations
	Aerococci	Total streptococci	
Occupied schoolrooms	6.92	1.16	763
Boot and shoe factories	2.00	0.52	23
Large clerical offices	0.83	0.15	90

The organisms are also very common in floor-dust from occupied places, on clothing, and in dust from yards and streets in London. In all these sites it is probable that they constitute some 5–10 % of the total aerobic flora that will grow on ordinary nutrient media at 37°. In the schoolrooms we have been able to show that the count of the aerococci in the air varies to some extent with the amount of activity by the occupants.

We have not been able to discover any obvious human source of the aerococci found in the environment. Organisms of this sort are present only in very small numbers, if at all, in the upper respiratory tract of normal persons, and they are by no means common in faeces. They may be present in rather small numbers on normal skin. We have not made any systematic search for aerococci on animals, but their frequency in occupied rooms from which animals are largely excluded, and on clothing, suggests that this is not a likely source.

DISCUSSION

Despite the large numbers of the organisms which we describe as aerococci that are present in the environment, they do not appear to have been well described in the literature. Buchbinder, Solowey & Solotorovsky (1938), who studied bacteria from air samples in occupied places, described what we presume to be the same organisms as 'putative streptococci', largely because of the similarity of their fermentation reactions to those of typical streptococci. On the other hand, Rabl & Seelemann (1949) described similar organisms from various lesions in humans as 'diplokokken', and seem to have regarded them as distinct from streptococci. Duguid & Wallace (1948) noted α -haemolytic micrococci as common in bacteriological samples from clothing, and Bourdillon, Lidwell & Lovelock (1948) found micrococci on crystal-violet blood agar plates, which were a source of confusion in a search for α -haemolytic streptococci in air samples. A number of similar organisms were deposited in the National Collection of Type Cultures by Dr C. L. Hannay, who had isolated them from dairy equipment and had noted their extreme resistance to disinfection by hypochlorite.

Several extensive studies of enterococci have been reported (e.g. Houston, 1905; Dible, 1921), and it might be thought that aerococci would have been included in these descriptions. However, we can find no direct reference to organisms with the characteristics that we describe. This is partly due to the fact that many of the tests by which we distinguish aerococci from enterococci are of relatively recent introduction. Moreover, in faeces, which were the source of most of the strains studied by Houston and by Dible, aerococci are not common, and any worker studying streptococci would tend to discard organisms that, microscopically, resembled micrococci rather than streptococci. The organisms were brought to our notice when we were studying the flora of the air in which they are common, and were employing a particular selective medium. We feel that when aerococci have been noticed in the past, they have probably been regarded as morphologically atypical enterococci. It is the fact of having handled so many cultures that gives us some confidence in describing them as different from enterococci.

The reasons for excluding the aerococci from the genus *Staphylococcus* were given by Shaw *et al.* (1951). The problem is to decide whether they are sufficiently closely related to the streptococci, particularly enterococci, to be classed with them. The absence of catalase production would suggest that they might be streptococci, but morphologically they do not resemble streptococci. The morphological criterion is admittedly unsatisfactory and could not be given much weight had our experience of the organisms been limited to the examination of a few strains, or of old laboratory strains. But during the last few years we have examined many thousands of films made from colonies, and many hundreds of blood agar cultures. We have no doubt that the aerococci form a group worthy of separate recognition, and we do not feel that they resemble the streptococci sufficiently to be classed with them.

It remains to be shown how the aerococci differ from species of *Pediococcus* Balcke, which Shimwell (1948) regarded as streptococci. Pederson (1949) characterizes pediococci as Gram-positive, non-motile, catalase-negative cocci which tend to form packets of four but which may occur as single or paired cells. They are microaerophilic, produce a final pH value of 3.25–3.4 in glucose broth, do not reduce nitrates to nitrites or liquefy gelatin. Aerococci differ from pediococci in that they do not normally form packets, they are not microaerophilic, and they do not form so much acid in glucose broth (final pH value 5.0–5.6). Pederson's cultures of pediococci seldom utilized mannitol; Shaw *et al.* found 69% of their α -group (aerococci) fermented this sugar, as did five of the twelve strains recorded in Table 1. Raffinose is fermented by nearly all pediococci but by only a third of aerococci. In addition aerococci tolerate crystal violet and potassium tellurite, which pediococci do not, and they produce marked greening on blood agar.

We are indebted to Dr B. C. Hobbs for the supply of staphylococcal antisera.

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EXPLANATION OF PLATE

Fig. 1. Colonies of aerococci on blood agar after 24 hr. incubation at 37° ($\times 4$).

Fig. 2. Aerococci grown in nutrient broth for 24 hr. at 37° and stained by Gram's method ($\times 2000$).

(Received 5 December 1952)

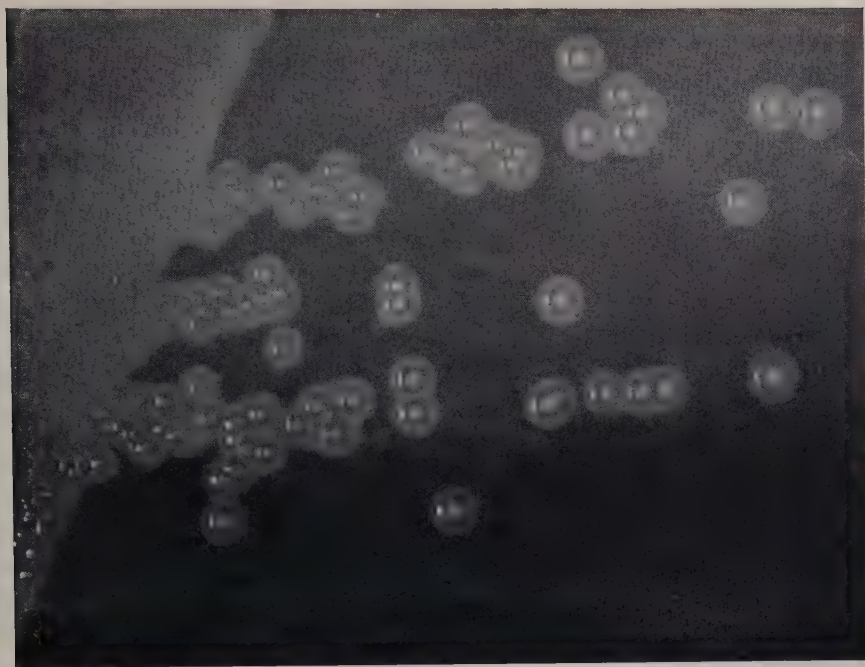


Fig. 1

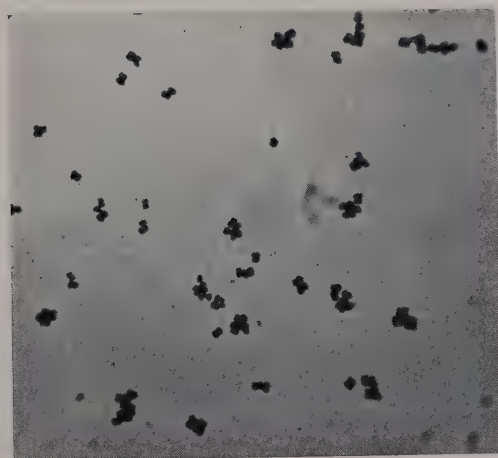


Fig. 2

R. E. O. WILLIAMS, A. HIRCH & S. T. COWAN—*AEROCOCCUS*, A NEW
BACTERIAL GENUS. PLATE 1

EVANS, D. G. & WARDLAW, A. C. (1953). *J. gen. Microbiol.* 8, 481-487.

Gelatinase and Collagenase Production by certain Species of *Bacillus*

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SUMMARY: Fifty-three strains from six species of the genus *Bacillus* were tested for their ability to produce gelatinase and to disintegrate the collagen matrix of bone. Some strains of *B. cereus* and *B. anthracis* produced a true collagenase which brought about complete disintegration of the collagen substrate, while strains of *B. subtilis*, *B. licheniformis*, *B. brevis* and *B. megaterium* were either completely inactive or produced only partial disintegration. Collagenase production was always accompanied by gelatinase production but the reverse was not the case. Concentrated culture filtrates of *B. cereus* were shown to contain collagenase and gelatinase, whereas those of *B. subtilis* contained gelatinase only.

In a previous communication (Evans & Wardlaw, 1952) we described some of the properties of proteolytic culture filtrates from a strain of *Bacillus subtilis*. The culture filtrates were highly active in liquefying gelatin and in disintegrating hide powder which is a denatured form of skin collagen. The filtrates were, however, unable to break down undenatured tendon collagen either in the form of thin slices or thin sheets prepared from soluble collagen by the method of Delaunay, Guillaumie & Delaunay (1949). *Bacillus* strains have, however, been shown to attack the collagen-like matrix of tooth dentine (Evans & Prophet, 1950), and this has led us to extend our previous investigation. We have now tested the ability of a number of bacillus strains to produce collagenase, using as substrate a finely divided preparation of decalcified bone. This preparation was not regarded as a pure substrate but was nevertheless a rich source of native collagen. The strains were also examined for their ability to produce gelatinase, and a study was made of the relationship between this enzyme and collagenase.

MATERIALS AND METHODS

Preparation of collagen substrate. Limb-bone shafts from adult rabbits were freed from marrow and connective tissue and dried over P_2O_5 . After preliminary fragmentation the bone was ground to a fine powder in a low-temperature ball mill as used by Evans & Prophet (1950) for the preparation of powdered dentine. The powder was decalcified by suspension in a large volume of 0.2N-HCl for 1 hr. and then centrifuged. The deposit was washed free from acid by resuspending in water and centrifuging several times; the final suspension was dialysed against running water and dried from the frozen state to yield a very finely divided preparation of decalcified bone.

Test for collagenase production. The decalcified bone powder was sterilized by exposing thin layers to ultra-violet radiation. A saline suspension of the powder was mixed with melted 3% agar in saline which had been cooled

below 45° and the mixture poured into Petri dishes. For each plate 10 ml. of agar and 20 mg. of decalcified bone powder were used. Disks 6 mm. in diameter were cut from the agar plate with a sterile cork-borer and transferred to tubes containing 10 ml. sterile 1 % Evans peptone in saline at pH 7.6. Two disks were placed in each tube. The tubes of medium after the addition of the disks were incubated for 1 week to check sterility; they were then inoculated with the bacillus strains listed in Table 1 and incubated at 37°. With aseptic precautions one disk was removed from each culture after 3 days, the other disk after 5 days and each examined microscopically at $\times 15$ magnification. The degree of disintegration of the collagen particles was determined by comparison with a control disk from a tube of uninoculated medium and was estimated as none, partial or complete (Pl. 1). No attempt was made to describe grades of partial clearing.

With some of the strains the collagenase activity of culture filtrates was titrated. Serial dilutions of the filtrates were made in normal saline, and to each was added one collagen-agar disk. The tubes were incubated at 37° for 18 hr. and the end-point taken as the highest dilution which gave complete clearing of the disk. The reciprocal of the end-point dilution gave the number of minimal collagenase doses/ml. (m.c.d./ml.).

Test for gelatinase production. The same cultures which were being tested for collagenase activity and which contained collagen-agar disks, were also tested for their gelatinase activity. After 2 and 5 days of incubation, 1 ml. samples of culture were removed and the gelatinase activity titrated by the setting-time method (Evans & Wardlaw, 1952). Serial dilutions of culture were made in saline, and to each was added an equal volume of 5 % gelatin solution. This concentration of gelatin we found more sensitive than the 10 % solution used previously. The mixtures were incubated for 1 hr. at 37° and then cooled in ice water. The time taken for each mixture to set was determined and the end-point taken as that dilution which gave the same setting time as a control solution of 1.5 % gelatin alone. The activity of a culture was expressed as the number of minimal gelatinase doses/ml. (m.g.d./ml.) which was the reciprocal of the end-point dilution. Culture filtrates were titrated in a similar manner.

Strains. The fifty-three strains used are given in Table 1. They were obtained as named species from the National Collection of Type Cultures (NCTC), from the Culture Collection of the Wellcome Research Laboratories (CN) and from our own departmental collection (DBM). In addition, Dr G. P. Gladstone supplied us with three strains of *B. anthracis*—M36, Sterne and NPA. We accepted the identity of the strains as received and from the first cultures prepared fresh batches of freeze dried cultures.

RESULTS

Gelatinase and collagenase in cultures

The results of the tests on the fifty-three different strains are shown in Table 1. Species were found to differ considerably in their ability to produce gelatinase. *B. anthracis* and *B. subtilis* were the most active, some strains of which gave titres of 100–300 m.g.d./ml. after 5 days, whereas no strains of *B. brevis* and

Table 1. *The ability of fifty-three Bacillus strains from six different species to produce gelatinase and collagenase*

<i>Bacillus</i> species and strain no.		Gelatinase activity (m.g.d./ml.)		Collagenase activity Degree of disintegration of collagen	
		2 days	5 days	3 days	5 days
<i>B. subtilis</i> :	NCTC 3610	120	90	0	+
	NCTC 6276	30	60	0	0
	NCTC 6346	2	4	0	0
	NCTC 7241	50	50	0	+
	CN 788	30	25	0	+
	CN 789	12	18	0	+
	CN 808	4	10	0	0
	CN 831	140	130	+	+
	CN 1508	25	50	0	+
	CN 1576	25	30	0	0
<i>B. licheniformis</i> :	NCTC 962	2	5	0	+
	NCTC 1024	1	7	0	+
	NCTC 1025	2	6	0	0
	NCTC 1026	5	11	0	0
	NCTC 1027	5	8	0	0
	NCTC 1097	5	8	0	+
<i>B. cereus</i> :	NCTC 6349	8	6	++	++
	CN 753	<1	6	0	0
	CN 2194	10	45	+	++
	CN 2484	2	16	++	++
	CN 2501	10	16	++	++
	CN 2503	25	70	++	++
	CN 2504	10	60	++	++
<i>B. cereus</i> var. <i>mycoides</i> :	NCTC 926	.	3	0	0
	NCTC 6093	.	1	0	+
	CN 1409	10	20	0	0
	CN 1541	8	15	+	+
	CN 2195	4	10	0	0
	CN 2495	8	25	+	++
	CN 2500	<1	8	0	++
	CN 2521	2	5	0	+
	NCTC 1712	<1	3	0	0
<i>B. anthracis</i> :	NCTC 2620	<1	3	0	+
	DBM 121	<1	5	0	+
	DBM 122	20	300	0	++
	DBM 123	20	250	0	++
	M36	<1	70	0	++
	Sterne	<1	70	0	++
	NPA	<1	3	0	+
	NCTC 7577	.	<1	0	0
<i>B. brevis</i> :	CN 2904	.	<1	0	0
	CN 2918	.	<1	0	0
	CN 2922	.	<1	0	0
	CN 2934	.	<1	0	0
	CN 3005	.	<1	0	0
	CN 3006	.	<1	0	0
	NCTC 2605	.	<1	0	0
<i>B. megaterium</i> :	NCTC 5636	.	<1	0	0
	CN 737	.	<1	0	0
	CN 1759	.	<1	0	0
	CN 2496	.	<1	0	0
	CN 2529	.	<1	0	0
	CN 2623	.	<1	0	0

A titre of <1 means that the undiluted culture showed no activity. Disintegration of collagen: 0=none; +=partial; ++=complete.

NCTC=National Collection of Type Cultures, Colindale, London, N.W. 9.

CN=Wellcome Research Laboratories, Beckenham, Kent.

DBM=Department of Bacteriology, Manchester.

B. megaterium showed detectable activity. The strains within a single species also showed marked differences in gelatinase production. With *B. subtilis* strains, for example, the titres ranged from 4 to 130, and with *B. anthracis* there were even greater differences, from 3 to 300. With many strains the gelatinase activity at 2 and 5 days was similar, although in some cases, as with *B. anthracis* and *B. cereus*, the 5-day cultures showed a considerably higher titre. Although it was not possible with some of the strains to detect gelatinase by the titration method, this did not imply that the strains were entirely devoid of the ability to produce the enzyme. It was possible to detect gelatinase action by these strains in gelatin stab cultures incubated for long periods.

The collagenase tests showed that some strains produced complete disintegration of the collagen particles in the disks while other strains were completely inactive. The greatest activity was shown by *B. cereus*; of the seven strains tested, complete disintegration was shown by five of them in 3-day cultures and by six in 5-day cultures. The *B. anthracis* strains were also able to produce complete disintegration, although they were not so rapidly active as those of *B. cereus*. None of the eight strains of *B. anthracis* showed activity in 3-day cultures, but after 5 days of incubation four of them had produced complete disintegration and three partial disintegration. Many of the strains of other species showed partial effects, but complete disintegration was given only by two strains of *B. cereus* var. *mycoides*. No detectable collagenase activity was shown by any of the strains of *B. brevis* or *B. megaterium*.

It was evident that in the group as a whole there was some correlation between collagenase and gelatinase production. Those strains which produced collagenase always produced gelatinase, and those which showed no gelatinase activity were also inactive towards collagen. The correlation was, however, by no means complete, for gelatinase production was not always accompanied by collagenase production. Further, with those strains which produced both enzymes, there was no quantitative correlation between gelatinase activity and rapidity with which collagen was disintegrated. This lack of association between the two enzyme activities was most apparent with strains of *B. subtilis* and *B. cereus*. For example, strain NCTC 7241 of *B. subtilis* had a gelatinase titre of 50 but showed no disintegration of collagen at 3 days. On the other hand, strain NCTC 6349 of *B. cereus* produced complete disintegration of collagen at 3 days, although the gelatinase titre was only 8. This strongly suggested that the proteolytic enzymes in the two cultures differed considerably in their substrate specificity, and in order to obtain more conclusive evidence of these differences further tests were made with concentrated culture filtrates.

Gelatinase and collagenase in filtrates

Concentrated culture filtrates were prepared from two strains of each of *B. subtilis* and *B. cereus* by the method we previously used (Evans & Wardlaw, 1952). Cultures were filtered after 3–4 days growth and the filtrates precipitated by adding 500 g. ammonium sulphate/l. The precipitate was dialysed

to remove ammonium sulphate and then dried from the frozen state. To be tested for enzyme activity the dried preparations were dissolved in saline to give approximately 1% (w/v) solutions. Each preparation was titrated for gelatinase and collagenase, and the results are given in Table 2. To facilitate comparison of the two activities, the gelatinase titres of *B. cereus* have been adjusted to equal those of *B. subtilis* and the collagenase titres scaled accordingly. Neither of the *B. subtilis* filtrates, although highly active in liquefying gelatin, were able to bring about complete clearing of the collagen-agar disks. The two *B. cereus* filtrates, on the other hand, with the same gelatinase activity as those of *B. subtilis*, were able completely to clear the disks even at dilutions of 1/260 and 1/64. These results are in agreement with those obtained in the tests with cultures and clearly show that the proteolytic enzymes of these two species differ in their substrate specificity.

Table 2. Comparison of the gelatinase and collagenase activities of culture filtrates of *Bacillus subtilis* and *B. cereus*

Filtrate	Gelatinase titre (m.g.d./ml.)	Collagenase titre (m.c.d./ml.)
<i>B. subtilis</i> : NCTC 3610	300	< 1
CN 831	300	< 1
<i>B. cereus</i> : NCTC 6349	300	260
CN 2504	300	64

The results also suggest that with *B. cereus* two enzymes are involved in attacking gelatin and collagen. The two *B. cereus* filtrates attacked gelatin to the same titre but there was a four-fold difference in their collagenase titres. If the enzyme which attacked gelatin was also responsible for the breakdown of collagen, then it would have been expected that the collagenase titres of the two filtrates would have been the same. The fact that they were considerably different strongly suggests that two independent enzymes were present in the filtrates, one which attacked collagen and possibly gelatin, and one which attacked gelatin only.

The nature of the collagen substrate

It is well known that collagen can undergo changes in its susceptibility to enzyme action. We found that heating a watery suspension of decalcified powdered bone at 65° for 10 min. considerably increased its susceptibility, so that it was completely disintegrated by enzyme preparations of *B. subtilis* which were unable to attack the unheated material. On the other hand, the bone powder was made completely resistant to enzyme action by treating it with low concentrations of formaldehyde or tannic acid. Not only did this treatment make the collagen resistant to bacillus collagenases but also to the more rapidly acting collagenase of *Clostridium histolyticum*. In view of this, care was taken in preparing the bone substrate to maintain the collagen in its unaltered native form. It was not possible to use untreated powdered bone as the collagen substrate, for bone in its calcified form was found to be completely unaffected by bacillus and clostridium collagenases. A similar observation

was made by Evans & Prophet (1950) with the collagen-like matrix of dentine. It was therefore necessary to decalcify the bone and 0.2N-HCl was used for this purpose. This treatment, apart from removing the calcium salts and thereby exposing the collagen matrix to enzyme attack, probably had little or no denaturing effect on the collagen, for it was found that bone powder decalcified with phosphate buffer at pH 6.0, a mild treatment hardly likely to denature the collagen, was just as sensitive to enzyme attack as bone decalcified by 0.2N-HCl.

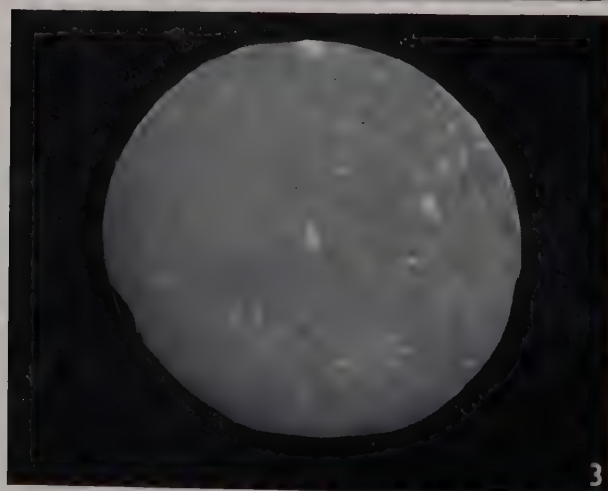
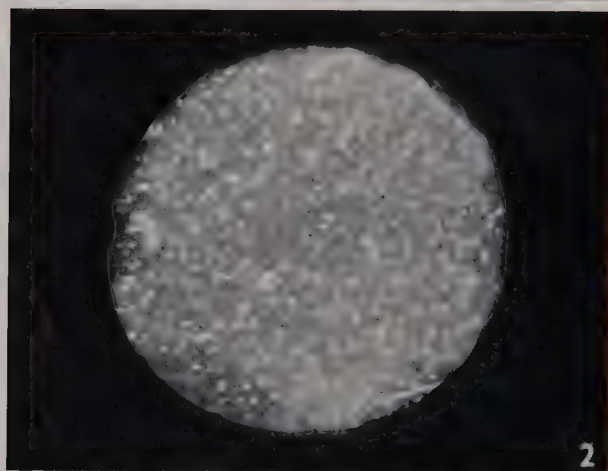
An experiment was also made which showed that the method we used for sterilizing decalcified bone powder by ultra-violet radiation did not alter its susceptibility to enzyme. Titrations of collagenase preparations made with agar disks containing irradiated decalcified bone powder gave the same end-points as titrations made with disks containing powder which had not been exposed to radiation.

As a result of these tests we considered that the substrate we used was native collagen which had not been denatured during its preparation. If this be accepted, then those bacillus strains which were able to disintegrate this substrate were true collagenase producers.

DISCUSSION

Although many bacteria are known to attack denatured collagen in the form of gelatin, relatively few species have been shown to break down native collagen. Our experiments indicate that some strains in the genus *Bacillus* are able to produce a true collagenase. It is possible that we have been able to detect this enzyme because of the finely divided form of substrate we have employed. The substrates commonly used in testing collagenase action are thin slices of tendon or thin sheets of precipitated collagen ('collagen paper'). These substrates are comparatively insensitive indicators and are not disintegrated by such high dilutions of enzyme as is collagen powder prepared from bone. With our most potent concentrated filtrates from *B. cereus*, we showed that agar disks containing collagen powder as indicator were at least 30 times more sensitive than collagen paper. Similar results were obtained with the collagenase of *Cl. histolyticum*. Decalcified bone we have found can be prepared as a very fine powder, and thus when treated with enzyme is intimately exposed to its action. It is possible that the collagen-agar disk method may be of value in detecting unsuspected collagenase production by other bacterial species.

It is not yet known whether the bacillus collagenases are of the same kind as those produced by *Cl. welchii* type A (Oakley, Warrack & van Heyningen, 1946) and *Cl. histolyticum* (Oakley & Warrack, 1950). The clostridium enzymes are able to break down with comparative ease the more resistant collagenase indicators such as collagen paper and even pieces of skin. This is due to the fact that they are produced in high concentration in culture and does not necessarily imply that they are of a different kind from the collagenases of the bacillus group. The collagenases of the two groups do, however, appear to differ in the rate at which they act on the substrate. We have shown, with



D. G. EVANS & A. C. WARDLAW—GELATINASE AND COLLAGENASE OF
BACILLUS SPP. PLATE 1

collagenase preparations from each group, containing the same number of m.c.d./ml., that the disintegration of bone powder is completed by the clostridium collagenases in less than half the time taken by those of the bacillus group.

The experiments have also shown that the collagenase activity of bacillus strains is not dependent on their gelatinase activity. Some strains with low gelatinase activity are highly active in disintegrating collagen, whereas others with high gelatinase activity have little or no effect on collagen. Even in the case of those culture filtrates of *B. cereus* which actively attack both gelatin and collagen, the results indicated that two independent enzymes are involved, one which attacks collagen and possibly gelatin, and one which attacks gelatin only. In this respect there is an analogy with the collagenase (κ -antigen) and gelatinase (λ -antigen) of *Cl. welchii* (Oakley, Warrack & Warren, 1948). The analogy, however, is by no means complete, for we have been unable so far to obtain conclusive evidence that either the gelatinase or the collagenase of *B. cereus* is antigenic. Further attempts are being made to obtain antibodies to these enzymes, for we consider that if it be possible to use serological tests, the proteolytic enzymes of this group will then be more clearly differentiated.

We should like to express our thanks to Mr H. Proom and Dr G. P. Gladstone for strains and to Dr G. H. Warrack for rat collagen paper. One of us (A.C.W.) wishes to acknowledge the receipt of a Scholarship from the Medical Research Council.

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EXPLANATION OF PLATE

Collagen powder, suspended in agar disks, showing different degrees of disintegration after exposure to bacillus cultures. (1) no disintegration, (2) partial, (3) complete. $\times 9$.

(Received 17 December 1952)

[The editors of the Journal of General Microbiology accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from authors.]

THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its Fifteenth General Meeting at Birmingham University on Tuesday and Wednesday, 23 and 24 September 1952. The following communications were made:

COMMUNICATIONS

Culture Study of an Unstable Strain of *Aspergillus niger*. By J. L. YUILL
(John and E. Sturge Ltd., Birmingham)

A. niger, strain 868, isolated from the outer scales of a Spanish onion, has given rise spontaneously to two different mutants, 868T and 868B. The conidial heads of 868 itself are practically black; those of mutant T are of a light tan colour and of mutant B, dark brown.

Mutant T appears to be stable, has substantially the same growth rate and acid-producing capacity as the parent 868, with which it readily forms heterokaryons. It is a colour mutant, analogous to Schiemann's *A. cinna-momeus*.

Mutant B, on the other hand, is unstable, has a lower growth rate than 868—as measured by the radius of its colony—and has a greatly reduced acid-forming capacity. The conidia of B, besides being lighter in colour than those of the parent 868, show certain morphological differences by which they can usually be recognized under the microscope. Point inoculations from single spore-heads of B produce, in the great majority of cases, relatively slow-growing brown colonies. These invariably, however, have given rise, sooner or later, to one or more black sectors of higher growth rate. Single head subcultures from such sectors give black colonies apparently identical with 868 in morphology, growth rate and acid-forming capacity, whilst subcultures from spore-heads in the brown area give brown colonies, again sectoring black.

No instance of a black colony giving rise to brown sectors has been met with, although one or two isolated brown spore-heads have appeared again in a black colony.

In surface fermentations on molasses the mutant B develops heavier mycelial felts than those of 868.

Observations on the Culture of *Leptospira* on Solid Medium. By J. W. CZEKALOWSKI and J. W. MCLEOD (University of Leeds)

Some Observations on Bacterial Flagella. By JOYCE B. GRACE (Department of Bacteriology, University of Birmingham)

The flagella of certain large *Spirilla* are compound structures composed of a large number of individual fibres, each of which is considerably finer even

than other bacterial flagella. The complete compound flagellum is readily visible by ordinary microscopic methods. The evidence suggests that these fibres arise in bunches in the cytoplasm and, whereas in other bacteria it appears that each flagellum arises from an individual blepharoplast, each of these bunches appears to arise from a single basal structure. It has been suggested that this type of flagellum in these primitive bacteria provides a connecting link between the typical bacterial flagellum and the more complex flagella of other micro-organisms.

The Nucleus and Ectoplasm of Bacteria. By M. G. GOOD (*London*)

Bacteriophages of Lysogenic Strains of *Pseudomonas pyocyanea*. By LOIS DICKINSON (*Boots Pure Drug Co., Ltd., Nottingham*)

The two types of plaques, P18 and P48, appearing on assay plates of filtrates of lysogenic culture C7X of *Ps. pyocyanea* were investigated further. Diluted filtrates passaged on an indicator culture several times yielded only a lytic type plaque, Px, with secondary bacterial growth resistant to Px but still sensitive to serologically distinct phages. The lytic plaques of C7X preparations were similar to Px. The hazy plaques, P48, always without a lytic zone, never yielded pure P48 plaques on passage; about 1/1000 was lytic. P48 also gave lytic mutants in latent period experiments. Serologically, the faint and lytic type plaques were distinct. P48 preparations gave rise to lysogenic cultures when spotted on the indicator strain; the cultures produced were resistant not only to P48 but to Px, whereas cultures derived from Px were still sensitive to P48.

A phenanthridinium compound prevented the action of both P48 and Px on the indicator culture when given within a few minutes of infection. Results were discussed in the light of current views on lysogenesis.

Observations on Streptococcal Bacteriophage. By W. R. MAXTED (*Colindale*)

The prevention of the formation of the transient hyaluronic acid capsule of group A haemolytic streptococci, by the inclusion of hyaluronidase in the growth medium, enhances the action of streptococcal bacteriophage.

Strains surviving passage through bacteriophage-containing medium are, unlike their parents, grossly mucoid. An apparent loss of T antigen in these survivors appears to be due to surface interference of agglutination and absorption, presumably by this mucoid material. One strain showed increased M antigen content and an ability to survive in normal blood not possessed by the parent strain. Mucoid colonial appearance, possession of M antigen and the ability to survive in human blood are characteristic features of virulent streptococci.

The Active Immunization of Mice against an Intracerebral Infection of *Haemophilus pertussis* Organisms by Intranasal Instillation of *H. pertussis* Antigens. By ANNIE M. BROWN (*Serum Research Institute, Carshalton*)

Nine mixed antigens were chosen to cover the following factors present in living cultures of *H. pertussis*: (1) substances increasing capillary and tissue permeability; (2) necrotizing properties; (3) specific antigens of large particle size; (4) specific antigens of small particle size. Active immunity was demonstrated by an intracerebral dose of *H. pertussis* organisms in mice such that either antibacterial antibody or antitoxin or both would give protection. This was found to be 40,000 organisms of the culture used.

Only two of the mixed antigens, a vaccine containing 15 % of a supernatant toxin and a vaccine containing 15 % of a supernatant toxoid, have all the factors present in living culture, and these two alone after intranasal instillation gave a significant immunity in mice to an intracerebral infection of *H. pertussis*. This resemblance to the intranasal instillation of non-lethal doses of living culture stimulates the search for a factor in supernatant toxin enabling the invasion of the body with a sufficient number of organisms to develop protective antibody.

Three-dimensional Patterns of Antigenic Modulation of *Haemophilus pertussis*, *H. paraptussis* and *H. bronchisepticus*. By B. W. LACEY (*Westminster Medical School, London*)

Variation of the ionic composition of the medium will induce, in the course of 24–72 hr., gross reversible changes in the antigenic structure of *H. pertussis* (Lacey, 1951). No selection is involved. Similar modulations are inducible in most strains of *H. paraptussis* and *H. bronchisepticus*. Each ion has a measurable influence which varies with temperature of incubation and tends to one or other stable antigenic state (major mode). Variation of ionic strength alone has no effect. A number of ions has been arranged in a pattern with a temperature scale. From this may be calculated the amounts of any chosen salts needed to induce a given antigenic mode at a given temperature. In all three species two major modes are inducible. Each mode has a distinctive agglutinability, absorbing power and antigenicity. With *pertussis* a third unstable form (minor mode) usually occurs at the inflexion between major modes. *H. bronchisepticus* can be grown with or without flagella in either of its major somatic modes. The variation of agglutinability with temperature and variation in the proportions of sodium, magnesium, chloride and sulphate ions has been examined in members of the three species. Three-dimensional diagrams of the results show that the same ionic mixture has an equivalent influence on different species at different temperatures. With any given ionic composition the temperature needed to induce a standard (iso) antigenic state rises in the order: *paraptussis* < *pertussis* < *bronchisepticus*. Different strains

have different iso-antigenic temperatures but none has been found so far to overlap the species limits. Four types of stable mutants have been found:

- (1) Non-flagellated with normally modulating soma.
- (2) Modulating from either normal major mode to a new (mutant) mode.
- (3) Non-modulating form of either major mode.
- (4) Non-modulating form of a new (mutant) mode.

Normal modes are smooth; mutant modes may be rough or smooth.

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Inhibition of Growth of Organisms of the Pleuropneumonia Group by Antibody. By D. G. FF. EDWARD and W. A. FITZGERALD (*Wellcome Research Laboratories, Beckenham, Kent*)

Four antisera, which had been prepared in rabbits against strains of pleuropneumonia-like organisms isolated from the human genital tract, when incorporated in culture media inhibited growth of the homologous strain and of other strains shown by the agglutination reaction to be serologically related. Other species of the pleuropneumonia group, antigenically different, were not inhibited. Antiserum diluted 1:1000 in the medium was inhibitory. Heating the serum to 56° for 60 min. did not affect its ability to inhibit growth. Inhibition thus appeared to be due to antibody acting independently of complement.

In serum broths, to which antiserum had been added, the homologous organisms failed to multiply but were not destroyed immediately. Living organisms could still be detected by subculture 24 hr. later.

Inhibition of growth of the homologous strain, but not of other strains, was also noted with antisera prepared against two P strains isolated from the bovine genital tract. Since growth of a bacterium occurs normally on a medium containing homologous antiserum, these observations suggest an interesting difference between organisms of the pleuropneumonia group and bacteria.

Bacterial Symbiosis in the Assassin Bug. By S. BAINES (*Department of Bacteriology, University of Birmingham*)

The symbiotic bacterium *Nocardia rhodnii* is found in the gut of the blood-sucking bug, *Rhodnius prolixus*, and remains quiescent, in the resting stage, throughout the early nymphal stages in the life cycle of the insect. During the later nymphal stages, preceding metamorphosis, the bacterium passed through its own life cycle, typical of the genus *Nocardia*, including the formation of branched filamentous forms, followed by fragmentation and reversion to the resting stages which persist throughout the adult stage of the insect. It is precisely the later stages of development, preceding metamorphosis, which are delayed or inhibited when the nymphs are freed of *N. rhodnii*.

It has been found that newly hatched *Rhodnius* nymphs, separated from stock cultures of insects before the first feed, develop free of the symbiotes, and devices such as egg sterilization were not necessary to obtain sterile nymphs.

The development of symbiote-free nymphs was observed in order to determine the nature of the factors normally supplied by *Nocardia rhodnii*, the lack of which inhibited metamorphosis.

These factors were found to be present in higher concentration in the blood of mice than of rabbits, since sterile nymphs were relatively little delayed when fed on mice as compared with complete inhibition of metamorphosis when fed on rabbits.

The restoration of the normal development of *Rhodnius* in the absence of *Nocardia rhodnii* was achieved by increasing the concentration of certain B group vitamins in their diet, by injection of the vitamins into mice before feeding the insects.

It has not, so far, been established whether these B group vitamins are solely responsible for the difference in development rates of symbiote-free *Rhodnius* nymphs fed on the blood of mice and rabbits.

Citrulline Breakdown by a Cell-free Extract of *Streptococcus faecalis*.

By V. A. KNIVETT (*M.R.C. Unit for Chemical Microbiology, University of Cambridge*)

An active cell-free extract was obtained by shaking a washed suspension of the cells (*c.* 20 mg. dry weight of cells/ml.) with Ballotini glass-balls in the Mickle Sonic Disintegrator at 50 cycles/sec. for 10 min. The Ballotini were filtered off and the preparation centrifuged at $2000 \times g$ for 10 min., leaving a clear translucent supernatant.

The enzymic breakdown of citrulline into ornithine, ammonia, and carbon dioxide was followed by measuring the liberation of carbon dioxide manometrically. The reaction was started by the addition of either adenosinediphosphate or adenosinetriphosphate, though adenosinediphosphate was found to be 3 or 4 times as active as adenosinetriphosphate. The rate of citrulline breakdown was related to the concentration of inorganic phosphate. When limiting concentrations of inorganic phosphate were used, the liberation of carbon dioxide was found to be accompanied by the disappearance of an equivalent amount of inorganic phosphate and the formation of a '10 min. acid hydrolysable' phosphate ester.

Samples taken during citrulline breakdown were examined by paper strip electrophoresis and photographed under ultraviolet light. Adenosinediphosphate had partly disappeared with the formation of adenosinemonophosphate and a new spot running ahead of adenosinediphosphate at the same rate as adenosinetriphosphate. The control mixture without citrulline did not show this spot, but it was present in the control in which adenosinediphosphate was replaced by adenosinetriphosphate. When ^{32}P -labelled phosphate was added to the incubation mixture, radioactivity was found to

be present in this spot. The formation of acethydroxamic acid from added acetate or the phosphorylation of added glucose in the presence of citrulline, ADP and inorganic phosphate was used as a test for the presence of an enzyme system synthesizing adenosinetriphosphate.

The Inhibition of Growth of Avirulent Strains of *Mycobacterium tuberculosis* by a Surface-active Polyoxyethylene Ether. By P. D'ARCY HART and R. J. W. REES (*National Institute for Medical Research, Mill Hill, London*)

The *in vitro* Activity of Isoniazid against *Mycobacterium tuberculosis*. By R. KNOX, M. B. KING and R. C. WOODROFFE (*Guy's Hospital Medical School, London*)

The MH37 Rv strain of *M. tuberculosis* is inhibited by less than 0.01 µg./ml. of isoniazid in Dubos's liquid medium if the end-point is read after 5–7 days' incubation at 37°. After longer periods the end-point shifts progressively. The apparent 'sensitivity' therefore depends partly on the length of incubation.

This phenomenon of 'escape' from the influence of the drug is due to at least two processes: (1) inactivation of the drug which occurs in uninoculated as well as inoculated media; (2) the emergence of resistant variants. These processes must be reckoned with in any attempt to decide to what extent isoniazid is bacteriostatic and to what extent bactericidal in different experimental conditions.

The shift in the end-point to isoniazid mentioned above appears to be retarded: (1) in the presence of streptomycin or P.A.S.; and (2) by increasing the temperature of incubation from 37 to 40°—despite the fact that in uninoculated media increase of temperature *accelerates* inactivation of the drug. Our experiments suggest that increase of temperature profoundly affects the response of the organisms to the drug either by making it more bactericidal or by suppressing the process by which resistant variants become selected. Drugs such as streptomycin and P.A.S. may affect the response of *M. tuberculosis* to isoniazid in a similar way.

The Action of Polymyxin on *Pseudomonas pyocyanea*. By B. A. NEWTON (*M.R.C. Unit for Chemical Microbiology, University of Cambridge*)

The leakage of purine and pyrimidine containing materials, pentose and phosphate from washed cells of a strain of *Ps. pyocyanea*, standing in distilled water has been observed. This leakage is reduced to a low level if the cells are suspended in 0.9 % saline instead of water. Treatment of cells in saline with polymyxin E results in the release of soluble cell constituents. In this respect polymyxin resembles other surface-active compounds. About 50 % of the purine and pyrimidine containing material, pentose and phosphate found in the supernatant from boiled cells is released from washed cells by polymyxin in 3–4 hr., the remainder being released at a slower rate during the

next 19 hr. The leakage from suspensions of 1.5 mg./ml. dry weight of cells is maximal in the presence of 100 μ g./ml. polymyxin, higher concentrations release materials at a slower rate.

Electron-photomicrographs of polymyxin-treated cells shows that the leakage of cell constituents is accompanied by morphological changes. The cytoplasm becomes more electron-dense and contracted away from the cell wall. Cells treated with 100 μ g./ml. of polymyxin for 4 hr. appear to have an accumulation of material around the outside of the cell.

Washed cells which have been grown on a casein-2 % glucose medium have a high endogenous respiration ($Q_{O_2}=40$). Low concentrations of polymyxin stimulate the endogenous oxygen uptake. Higher concentrations inhibit the endogenous respiration completely, but these cells are still capable of oxidizing glucose, the oxygen uptake corresponding to 2 atoms/molecule of glucose, and a 2:ketohehexonic acid accumulates. In the case of cells grown in the absence of glucose, the oxygen uptake in the presence of glucose and polymyxin is only 1 atom/molecule of glucose. No 2:ketohehexonic acid accumulates but paper chromatography shows the presence of gluconic acid.

The Action of Antibiotics on the Incorporation of Glutamic Acid into the Proteins of *Staphylococcus aureus*. By E. F. GALE (*M.R.C. Unit for Chemical Microbiology, University of Cambridge*)

If washed suspensions of *Staph. aureus* are incubated with glucose and ^{14}C -labelled glutamic acid (condition 1), radio-glutamic acid becomes progressively incorporated into the protein fraction of the cells. The rate and degree of incorporation are markedly increased if a complete mixture of the other amino-acids essential for growth are added to the medium (condition 2). Increase in the cellular protein can be demonstrated under condition 2 but not under condition 1. The incorporation is prevented in both cases by exposure of the cells to 100° , omission of glucose from the incubation mixture, or by addition of 0.01M-2:4-dinitrophenol. Concentrations of chloramphenicol, aureomycin or terramycin which inhibit growth of the organism also produce 90 % inhibition of the incorporation of glutamic acid occurring under condition 2 but produce only 6-25 % inhibition of that occurring under condition 1. Bactericidal concentrations of bacitracin and penicillin inhibit incorporation under condition 1 but have little or no action on that occurring under condition 2.

When cells are incubated in condition 1, accumulation of free glutamic acid occurs within the cells. As the cell protein becomes progressively more radioactive, the specific activity of the free glutamic acid within the cells progressively decreases. Inhibition of incorporation by penicillin is accompanied by increased specific activity of the free glutamic acid. It is suggested that the incorporation, that occurs in the absence of net protein synthesis, takes place as a result of exchange between free glutamic acid within the cells and glutamate-residues in the cell protein.

The Metabolism of Glutathione by Bacteria and Actions of Antibiotics thereon. By PAMELA J. SAMUELS (*M.R.C. Unit for Chemical Microbiology, University of Cambridge*)

A cell-free extract of *Escherichia coli* will synthesize glutathione. The extract has been prepared by grinding the organisms with alumina, and forms glutathione on incubation with glutamic acid, cysteine and glycine in the presence of adenosinetriphosphate, hexosediphosphate, coenzyme I, potassium phosphate and magnesium ions. The glutathione has been identified qualitatively by paper chromatography and quantitatively by the glyoxalase method of Woodward (1935), and kinetics of the reaction have been studied. None of the series of antibiotics penicillin, chloramphenicol, streptomycin, dihydrostreptomycin, neomycin, terramycin, aureomycin, gramicidin, polymyxin or bacitracin has any effect on the synthesis at concentrations of 100 $\mu\text{g./ml.}$

Glutathione is hydrolysed to its component amino-acids by washed suspensions and extracts of *Proteus vulgaris*. If phenylalanine, or certain other amino-acids, is added to the incubation mixture chromatographic examination shows that new materials are produced. A satisfactory identification has not been obtained, but the possibility that these are transpeptidation reactions since the new materials have the same R_F values as the γ -glutamyl peptides of the added amino-acids, is under investigation. Penicillin in equimolar concentration with the substrate inhibits hydrolysis and transpeptidation of glutathione by extracts of sheep kidney, and inhibits the breakdown of glutathione and formation of new material by *Proteus* suspensions. Terramycin in high concentration inhibits transpeptidation by kidney enzyme, and the formation of new material by *Proteus*. Chloramphenicol has no effect on either reaction.

REFERENCE

WOODWARD, G. E. (1935). *J. biol. Chem.* **109**, 1.

FILM

***Halobacterium halobium*.** By A. PIJPER (*Institute for Pathology, Pretoria*)

Halobacterium halobium is a new shape in Bacteria. This 'bacillus' is not cylindrical, but flat. It is also usually twisted along its long axis, and resembles a piece of ribbon to which a spiral twist has been given. The twist has to do with motility, the bacterium spins spirally when it moves. There are no flagella. Its flatness may have to do with its habitat, it lives in 80 % salt. Dark-ground microscopy was needed to bring out its curious shape, sunlight became essential to film it. Dried smears are useless for morphology. (Cf. Spruit & Pijper, in *Antonie van Leeuwenhoek*, 1952, p. 190.)

DEMONSTRATIONS

K. A. BISSET (*University of Birmingham*). 'Cytological appearances in bacteria.'

J. F. D. SHREWSBURY and G. J. BASSON (*University of Birmingham*). 'Demonstration of a large *Spirillum*.'

B. W. LACEY (*Westminster School of Medicine, London*). 'Solid models of the variation of the surface antigens of *H. pertussis*, *parapertussis* and *bronchi-septicus* with temperature of incubation and ionic composition of the medium.'

M. G. GOOD (*London*). 'The nucleus and ectoplasm of bacteria.'

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